PATENT NO EP (UK) 0334841

TRANSLATION OF EUROPEAN PATENT (UK) UNDER SECTION 77 (6) (a)

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IN THE MATTER OF

European Patent (UK)

No 0 334 841

I, Ralph David Waldman, of 50 Station Road, Fulbourn, Cambridge, England, do hereby declare that I am conversant with the English and German languages and that I am the translator of European Patent Specification, No 0 334 841 and certify that the following is a true translation to the best of my knowledge and belief.

Dated this 23rd day of December 1993

The present invention relates to the production, by genetic engineering, of plasmids and bacterial strains containing the gene tfdA, or a gene essentially identical to tfdA, on a short, exactly characterizable DNA segment. The novel plasmids and microorganisms are especially well suited for the production of 2,4-D-monooxygenase, as well as a starting product for the genetic transfer of the 2,4-D-degrading properties of this enzyme to various organisms (including the thus attainable 2,4-D-tolerance in genetically transformed plants).

2,4-D-Monooxygenase is an enzyme that catalyses in many 2,4-D-degrading organisms the first step in the metabolism of 2,4-dichlorophenoxyacetic acid (2,4-D). Among the 2,4-D-degrading organisms belong, in particular, soil bacteria, such as, for example Acinetobacter, Alcaligenes, Arthrobacter, Corynebacterium and Pseudomonas [compare, in this connection, G.R. Bell, Can. J. Microbiol. 3 : 821 (1957); J.M. Bollag et al., J. Agric. Food Chem. 16 : 826 (1968); R.H. Don et al., J. Bacteriol. 145 : 681 (1981); W.C. Evans et al., Proc. Biochem. Soc. Biochem. J. 57 : 4 (1954); W.C. Evans et al., Biochem. J. 122 : 543 (1971); R.P. Fisher et al., J. Bacteriol. 135 : 798 (1978); T.I. Steenson et al., J. Gen. Microbiol. 16 : 146 (1957); J.M. Tiedje et al., J. Agric. Food Chem. 17 : 1080 (1969); J.E. Tyler et al., Appl. Microbiol. 28 : 181 (1974); J.M. Tiedje et al., J. Agric. Food Chem. 17 : 1021 1969)]. They play a significant part in the detoxification of the soil and of the wastewaters from halogenated aromatic compounds which occur especially among the agriculturally utilized pesticides and herbicides.

In the group of 2,4-D-degrading wild-type bacteria, the strain Alcaligenes eutrophus JMP134 is the most well-known and best characterized. This strain harbours the plasmid

pJP4 having a size of about 80 kilobases and contains all of the genes important for degradation of 2,4-D (R.H. Don et al., loc. cit.). The plasmid pJP4 has also been isolated and characterized recently [R.H. Don et al., J. Bacteriol. 161 : 466 (1985)].

Five genes participating in 2,4-D degradation denoted as tfdB, tfdC, tfdE and tfdF, were localised by transposon mutagenesis and cloned in E. coli. R.H. Don et al., [J. Bacteriol. 161 : 85 (1985)] succeeded in attributing enzyme function to four genes by biochemical studies. However, thus far attempts have been unsuccessful regarding localizing of gene tfdA on the plasmid pJP4 or on a sub-fragment of the latter, and to isolate this gene and to clarify its structure.

It is now been possible, for the first time, thanks to the present invention to isolate, clone and characterize the gene tfdA. It has thus become possible to make this gene available for transfer to other organisms with the object of expressing the tfdA-encoded 2,4-D-monooxygenase in these organisms. The latter includes microorganisms, as well as higher organisms, such as, for example, plants.

The targeted transfer of tfdA to other microorganisms offers the possibility of broadening the spectrum of substances degradable by these organisms. As far as bacteria are concerned, transfer and expression of genes of the entire 2,4-D degradation has been disclosed in J. Bacteriol. 145 : 681 (1981) and in Arch. Microbiol. 134 : 92 (1983). However, no attempt has been made to express an isolated tfdA gene in gram-negative bacteria, such as Pseudomonas or Alcaligenes.

No known attempt has been made in connection with plants,

either. Metabolism of 2,4-D by several types of plants has been reported by various sources [Weed Science 24 : 557 (1976) and Z. Pflanzenphysiol. 110 : 395 (1983)]. In lower concentrations, this compound acts as an auxin-analogue plant hormone and therefore is utilized in cell culturing technique; at higher concentrations, it acts on the plant cell as well as on the entire plant in the manner of a growth inhibitor, which is the reason why it is utilised as a herbicide. In a haploid cell suspension culture of Nicotiana silvestris, adaptation to rising concentrations of 2,4-D brought about tolerance with respect to this synthetic growth compound based on an increased rate of metabolism [M.H. Zenk, 1974, Haploids in physiological and biochemical research, in Haploids in higher plants. Advances and potential; Proceedings of the First International Symposium, Guelph, Ontario, Canada, p. 339].

Since the tfdA gene mediates the cleavage of the side-chain of 2,4-D, the ability to inactivate 2,4-D could be transferred, with the help of the genes obtained from bacteria to all such plants for which a genetic manipulation of this type is possible. Methods for transferring foreign genes to plants and their progeny are already known for several types of plants (M. De Block, L. Herrera-Estrella, M. Van Montagu, J. Schell, and P. Zambryski, Expression of foreign genes in regenerated plants and in their progeny, EMBO J. 3 : 1681; as well as R.D. Shillito, M.W. Saul, J. Paszkowski, M. Muller, and J. Potrykus, 1985, High efficiency direct gene transfer to plants, Bio/technology 3:1099), and will in the future lead to greater applicability.

There are great hopes for the genetic transformation of plants, especially regarding the production of novel useful plants important to man [J.L. Marx, 1985, Plant

gene transfer becomes a fertile field, Science 230 : 1148]. In this context, the gene tfdA provides the availability of a novel, genetically transferable and in vivo selectable property in the form of tolerance against growth inhibitor, such as has only up till now been known for a few tolerances against antibiotics and herbicides [R.T. Fraley et al., 1983, Expression of bacterial genes in plant cells, Proc. Natl. Acad. Sci. USA 80 : 4803; and Nature 317 : 741 (1985)].

The present invention, first provides a heretofore unknown mutant using a known mutation method, from a bacterial strain capable of utilizing 2,4-D as the growth substrate, such as, for example, Alcaligenes eutrophus JMP134, the structural gene for 2,4-D-monooxygenase being inactivated in this mutant. This mutant is used, employing the known methods of DNA recombination in vitro, of transformation with recombinant DNA, and of conjugative transfer of DNA, for the selection of recombinant DNA containing tfdA or genes substantially identical to tfdA.

Another aspect of this invention relates to plasmids containing the gene tfdA or genes substantially identical to tfdA as well as plasmids containing parts of these genes including the promoter region.

The plasmids of this invention can be produced by digesting DNA from wild-type bacteria exhibiting genes for metabolizing 2,4-D or compounds similar to 2,4-D with restriction endonucleases and coupling the thus-obtained DNA fragments, with the aid of a DNA ligase, with a plasmid vector which latter has previously been converted into its linear form by the same restriction enzyme.

Among the thus-formed recombinant plasmids, the tfdA-

containing plasmids according to this invention can be identified by introducing the plasmids into bacteria strains from which then the tfdA-containing clones can be directly selected based on a tfdA-conveyed ability.

Such bacteria strains have the property of being able to exploit as sources of carbon and energy the products formed in an enzymatic reaction in vivo from 2,4-D-monooxygenase but not the substrates thereof.

Examples of strains having the aforementioned ability are the tfdA mutants according to this invention wherein all genes are active for degradation of 2,4-dichlorophenol, furthermore the strain Alcaligenes eutrophus JMP222 which exhibits degradation pathways for phenol, as well as Pseudomonas sp. B13 which can utilize 4-chlorophenol. However, a series of further bacterial strains which can degrade differently substituted phenols and are encountered primarily among the group of gram-negative bacterial, can conceivably be recipients for the selection and identification of cloned tfdA genes.

Preferred cloning vectors are plasmids having a wide host range, capable of replicative growth even in bacterial strains other than E. coli. However, plasmids capable of multiplying their DNA or parts of their DNA by integration into the genome of the host cell can likewise be used for this purpose.

A suitable process for introducing DNA into the living cells of the aforementioned bacterial strains is, first of all, the direct transformation of these strains insofar as methods therefore are known. Thus, transformation methods exist, for example, for Pseudomonas and related gram-negative bacteria [A.A. Mercer and J.S. Loutit, 1979,

Transformation and transfection of Pseudomonas aeruginosa: effects of metal ions, J. Bacteriol. 140 :37-42; A.M. Chakrabarty, J.R. Mylroie, D.A. Friello, and J.F. Vacca, 1975, Transformation of Pseudomonas putida and Escherichia coli with plasmid-linked drug-resistance factor DNA. Proc. Natl. Acad. Sci. USA 72 : 3647-3651]. In contrast thereto, a procedure which is applicable to all gram-negative bacteria and is substantially more effective is the transformation of an E. coli strain according to a method known from the literature with subsequent conjugative transfer of the plasmids into the respective bacterial strains.

Mobilizable plasmid vectors are preferably used for the conjugative transfer of cloned DNA. The genes required for transfer are made available in this case wither by so-called helper plasmids or by mobilizing strains specifically constructed for this purpose.

The plasmids containing tfdA can be isolated according to known methods as unequivocally definable chemical compounds from the aforesaid bacterial strains obtained directly by selection on suitable growth substrates or from clones of E. coli identified by the aforementioned method or by other conventional testing systems as regards expression tfdA.

The advantage of direct selection of tfdA-containing strains by a growth test resides, above all, in that it permits, in contrast to the previously known methods all of which are based on relatively expensive enzyme tests, the identification of a tfdA-containing plasmid among a very large number of various plasmids as they are produced with preference in the preparation of gene banks, i.e. the randomized cloning of DNA fragments of genomic DNA. The

advantage thus resides in broad applicability of the process, for cloning of tfdA genes is thus no longer restricted to wild-type strains carrying the gene tfdA on a readily isolable plasmid, but rather is made possible from almost all wild-type strains, even those containing the gene on a poorly accessible plasmid or on the chromosome.

The plasmids of this invention are obtained, for example, by cutting the plasmid pJP4, stemming from Alcaligenes eutrophus JMP134, on which the genes lie for degrading 2,4-D, with the restriction endonuclease HindIII, separating the thus-formed DNA fragments by electrophoresis, and linking the individual isolated fragments with the HindIII-cut and dephosphorylated vector pVK101. The mobilizing strain E. coli S17-1 is transformed with the recombinant DNA, and plasmid-containing strains are selected on a tetracycline-containing medium. From strains containing recombinant plasmids identified by restriction analysis, the plasmid DNA is transferred by conjugation to the above-mentioned tfdA mutant JMP134:Tn5-2, and expression of the cloned tfdA gene is confirmed by growth of the host strain on 2,4-D-containing minimal medium. In this way, the plasmid pVJH21 can be identified containing the gene tfdA on a HindIII fragment having a size of 21 kilobases, stemming from pJP4. The identity of the cloned fragment can be confirmed by isolation of the plasmid and subsequent restriction analysis.

The plasmids according to this invention can furthermore be prepared by subcloning from recombinant plasmids containing the gene tfdA. For this purpose, these plasmids are digested with one or several restriction endonucleases and the thus-formed fragments are linked with the aid of a DNA ligase with a vector plasmid that has been converted

into its linear form with the same restriction endonucleases. Plasmids that contain tfdA cam be selected from the thus-formed plasmids in the way described above.

Thus, for example, the plasmid pGJS3 can be produced by linking DNA fragments of the plasmid pVJH21, formed with SacI, with the SacI-cut vector plasmid pGSS33. Among the number of newly combined plasmids, those can be selected which contain an intact tfdA gene by transferring the recombinant plasmid DNA first by transformation into the mobilizing strain E. colin S17-1 and then from there by conjugation into the tfdA mutant JMP134:Tn5-2. By selection on 2,4-D-containing medium, plasmids are identified which contain a SacI insert having a size of 3 kilobases, the origin of which can be clearly traced back by restriction analysis to the HindIII fragment, having a size of 21 kilobases, from pVJH21, and thus to pJP4.

By subcloning DNA segments containing tfdA, those plasmids according to the invention can also be produced which differ in the type of plasmid vectors utilized, in dependence on pursued target of usage.

Thus, for example, the 3-kilobase SacI fragment from PGJS3 can be transcloned into the vector pKT231, thus forming the plasmids pKJS31 and pKJS32, offering as contrasted to pGJS3 advantages for the further characterization of tfdA due to a more favourable restriction map and the kanamycin resistance well expressed in many gram-negative bacteria. A preferred method for confirming tfdA expression by pKT231-derived plasmids as described hereinbelow is the conjugative transfer from E. coli S17-1 into the strain Alcaligenes eutrophus JM P222 with subsequent testing for utilisation of phenoxyacetic acid as the growth substrate. This system has the advantages over the tfdA mutants that

in this recipient strain the conjugative transfer takes place at higher efficiency and, in the kanamycin resistance, a further selectable marker is made available.

By subcloning, tfdA-containing DNA fragments can also be incorporated into expression vectors on which tfdA genes are expressed with the aid of a foreign (heterologous) promoter.

Thus, it is possible, for example, to incorporate the 2.8 kilobase sized SacI/SalI fragment, the 2.0 kilobase sized XbaI/SalI fragment into the expression vectors pT7-5 and PT7-6 directly adjoining a phage promoter by means of which gene expression can be activated in a controlled fashion with the aid of a promoter-specific RNA polymerase. The tfdA gene product expressed by the recombinant plasmids PTJSS'035, pTJS'B435 and pTJS'X535, the production of which will be described in Examples 8, 9 and 10, can be identified by specific labelling with radioactive methionine and subsequent gel electrophoresis. The system can furthermore be used for producing, in an E. coli strain, large amounts of 2,4-D-monooxygenase, which substantially facilitates purification and subsequent protein chemical and enzymatic characterization of the gene product as compared with its isolation from the wild-type strain whereby additional biotechnological usage possibilities become accessible to research.

Subcloning of tfdA-containing DNA fragments in phage vectors of the type of the M13 phage makes it possible to produce single-stranded DNA. The latter can be utilized for determining the base sequence according to the method of Sanger [F. Sanger, S. Nicklen, and A.R. Coulson, 1977. DNA sequencing with chain-terminating inhibitors, Proc. Natl. Acad. Sci. USA 74 : 5463]; it can furthermore serve

for the controlled mutagenesis of individual bases by means of oligonucleotides, a conventional method permitting the production of restriction scission sites in a gene or the alteration of the amino acid sequence. The insertion of restriction sites in genes broadens the possibilities of application of the latter because cutting sites are thereby created for the incorporation of foreign promoters and the linkage of gene fragments for the formation of fusion proteins, as they are required, for example, for the expression of prokaryotic genes in eukaryotes.

Subcloning of the 2.8-kilobase SacI/SalI fragment from pKJS32 into the phage vectors M13tg130 and M13tg131, as described in Example 16, represents a possibility for making the gene tfdA accessible to sequencing. By modification of the double-stranded DNA of the recombinant phages MJSS'030 and MJSS∇031, additional alterations can be performed on the insert-DNA.

The plasmids according to this invention can furthermore evolve by modification from tfdA-containing plasmids. By treating such plasmids with one or several restriction endonucleases, optional treatment with exonucleases, and subsequent reassembly of the DNA ends to an annular molecule, deleted plasmids are obtained, i.e. plasmids reduced in size by a specific DNA segment, which can contain a furthermore intact tfdA gene.

Thus, it is possible, for example, by the removal of defined DNA fragments from the plasmids pKJS31 and pKJS32, to shorten the insert contained therein, having a size of 3 kilobases, on one side, up to the XbaI scission site and, on the other side, up to the SalI scission site, without losing the activity of tfdA during this step. The

thus-produced plasmids pKJSB330, pKJS(X)630 and pKJS32RHΔS', described in Examples 4, 5 and 7, are capable of expressing tfdA gene activity. In this way the position of the gene can be delimited to an XbaI/SalI fragment of the size of 1.4 kilobases.

By the deletion disclosed in Example 16, of variously long DNA segments from the double-stranded forms of the recombinant phages MJSS'030 and MJSS'031 by means of exonuclease III, a number of phages can be produced wherein respectively different regions of the cloned DNA lie closest to the starting point for sequencing. The sequence of a 2-kilobase BamHI/SalI fragment can then be composed of the partial regions sequenced in overlapping relationship. From a knowledge of the base sequence, further properties of the tfdA-containing DNA can then be derived, such as, for example, the position and length of the encoding region and the position of restriction scission sites, which can be of considerable utility for the further use of tfdA.

By deletion of DNA fragments from tfdA-containing plasmids and, respectively, by subcloning DNA fragments, it is also possible to obtain further plasmids according to this invention which then merely contain parts of a tfdA gene. Thus, for example, by subcloning the 1.5-kilobase EcoRI/BamHI fragment from pKJS32 into pKT231, the plasmid pKJEΔB130 is obtained which merely contains the 5'end of tfdA, i.e. the promoter and the sequence coding for the N-terminus, and which is no longer capable of expressing an enzymatically active gene product. Parts of genes can be utilized for the construction of plasmids wherein the 5'end of a coding DNA, including its promoter, are linked in the same reading frame with the 3' end of another coding DNA and thus lead to expression of a so-called

fusion protein in those organisms recognizing the respective promoter.

Another possibility of modifying the plasmids of this invention resides in the insertion of a foreign DNA segment. The insertion can serve for the introduction of new functional DNA sequences, e.g. of restriction scission sites, promoters, resistance genes, translation and transcription stop signals. The insertion of a portion of a foreign gene sequence can also lead to the formation of fusion proteins.

One example of insertion of a foreign DNA is the insertion of the omega fragment into the BglII cutting site of the plasmid pTJS'X535 located in the centre of the coding region of tfdA (Example 11). With this fragment, translation stop codons are incorporated in all reading frames and transcription stop signals are incorporated together with a selectable resistance to antibiotics. As can be confirmed by specific radioactive labelling of the gene product as well as by in vivo enzyme test for 2,4-D-monooxygenase (Example 15), this results in the expression of a shortened, no longer enzymatically active protein by the recombinant plasmid pTJS'X535 omega.

Cloned fragments containing tfdA or parts of tfdA can serve for the detection of homologous DNA sequences and thus for finding tfdA genes in other organisms. For this purpose, these fragments are excised from the plasmids of this invention with the aid of suitable restriction enzymes, isolated, and labelled in accordance with methods known from the literature, for example by incorporation of radionuclides. By means of the conventional hybridization method, fragments can be identified in the entire DNA of an organism or in a gene bank produced therefrom, which

fragments exhibit homology with the tfdA. In this way, it is possible to recognize even among a population of various organisms those which contain such a tfdA-homologous sequence. The process can be utilized for tracking down novel 2,4-D-degrading organisms as well as for the discovery of tfdA genes in these organisms.

It is possible to transfer tfdA genes to other organisms either directly with the aid of plasmids of the invention insofar as they are suitable for this purpose, or after modification by means of conventional genetic engineering techniques. The transfer can serve the purpose of expressing the tfdA-coded 2,4-D-monooxygenase in these organisms and thus to imbue the latter with the property of being able to degrade 2,4-D or compounds similar to 2,4-D.

Thus it is possible, for example, by means of the conjugative transfer of tfdA-containing plasmids with a wide host range into various gram-negative bacteria, described in Example 14, to impart to these bacteria the property of converting 2,4-D or compounds similar to 2,4-D to the corresponding phenol. This, in conjunction with a degradation activity inherent in the respective strain, can lead to a broadening of the spectrum of compounds degradable by this strain. The strain Alcaligenes eutrophus JMP222 possesses, for example, genes for metabolizing phenol. The expression of tfdA thus conveys to this strain the entirely novel possibility of utilizing phenoxyacetic acid as the growth substrate. The strain Pseudomonas B13 can metabolize phenol as well as 4-chlorophenol, and receives, by tfdA, in an analogous fashion the capability of growing on phenoxyacetic acid and 4-chlorophenoxyacetic acid. Likewise possible is the degradation of variously substituted phenoxyacetic acids,

such as 4-chloro-2-methylphenoxyacetic acid, 2,4,5-trichlorophenoxyacetic acid, and similar compounds, by organisms containing tfdA and which are able to convert the corresponding phenols. Even though the thereby attainable broadening of the spectrum of degradation activities of an organism will not lead, in every case, to the creation of a novel property heretofore not realized in nature, it can yet serve for qualitative improvement insofar as strains can thereby be constructed having a degrading power that is higher as compared with wild-type strains, or strains having higher tolerance against possibly toxic substrates or products of a degradation pathway. Such strains would prove useful specifically under conditions prevailing in artificial systems, rather than in a natural environment, such as, for example, in clarification systems for the purification of industrial wastewater. An survey has recently been published regarding the performance attainable heretofore in the field of microbial degradation of halogenated aromatic compounds with the aid of newly constructed strains [D. Ghosal, I.-S. You, D.K. Chatterjee, A.M Chakrabarty, 1985, Microbial degradation of halogenated compounds; Science 228 : 135-142].

A further possibility of utilizing the gene tfdA resides in its transfer to plants. Methods for transformation of plants with foreign DNA are known in the literature and tested. Up till now, two methods can be distinguished. One uses the functions of the Ti plasmid from Agrobacterium tumefaciens; the other is based on the transformation of plant protoplasts with the aid of physical and chemical means and agents, such as electroporation, thermal shock and treatment with calcium chloride or polyethylene glycol. While transformation of plant cells by either method is always possible, in principle, the regeneration

of entire plants from individual cells or cell tissues is not achieved in every instance. At present, this represents the primary obstacle to the manipulation of monocotyledonous plants by genetic engineering.

A preferred objective of plant transformation with tfdA is the expression of the gene characteristic, ie. the 2,4-D-monooxygenase activity, in the transformed plant, thus enabling the plant to degrade the derivatives of phenoxyacetic acid, acting as an auxin-analogous plant hormones at low concentrations and as a growth inhibitor at high concentrations, to the phytochemically inactive phenols. Accordingly, this property would have significance as a selectable marker for genetically transformed plants.

In order for a prokaryotic gene, such as tfdA, to be expressed in plant cells, it must be coupled with the plant-specific signal sequences necessary for transcription and translation. It has been mentioned that this coupling can take place by the coincidence-steered integration of a gene into the genome of a plant transformed therewith. Prokaryotic genes, however, are preferably combined in vitro with the suitable plant-specific expression signals. A generally applicable method resides, for example, in linking the 5′ end of a structure gene, including the associated plant promoter with the coding sequence of a prokaryotic gene in such a way that a fusion protein is synthesized in a plant cell transformed therewith. This fusion protein consists of the N-terminal amino acids of the plant protein and a predominant proportion of the prokaryotic protein and, in its essential properties, corresponds to the prokaryotic protein [R.T. Fraley et al., 1983, Expression of bacterial genes in plant cells, Proc. Natl. Acad. Sci. USA 80 :

4803; J. Paszkowski and M.W. Saul, 1986, Direct gene transfer of plants, in S.P. Colowick and N.A. Kaplan (ed.), Methods in Enzymology 118 : 668]. A similar method utilizes solely the plant promoter, including a portion of the 5'-untranslated region of the transcribed m-RNA and makes do without the 5' end of the coding region of a plant gene. In the expression in plants, an ATG codon of the prokaryotic gene segment then serves as starting point for the translation. Analogously, the expression signals of plant viruses can be employed [N. Brisson and T. Hohn, 1986, Plant virus vectors; cauliflower mosaic virus. In S.P. Colowick and N.O. Kaplan (ed.), Methods in Enzymology, 118 : 659]. Vectors for the expression of prokaryotic genes in plants are known in the literature and are generally available.

A prerequisite for construction of a gene expressible in plants from a prokaryotic gene with the aid of the aforementioned vector plasmids resides in knowing the base sequence as present for the gene tfdA. Such knowledge permits exact localization of restriction cutting sites and thus makes it possible to link the various DNA segments in a base-accurate fashion. Furthermore, only such knowledge makes it feasible to perform controlled mutagenesis for the creation of novel, functionally significant DNA sequences, such as, for example, the introduction of novel restriction cutting sites.

The invention will be explained in greater detail below with reference to the figures and examples. In the examples, methods of genetic engineering have been utilized as they are generally known and well-tested in this field of art. Cloning of genes and analysis of gene structures were performed according to standard methods of genetic engineering as they have already been incorporated

into the textbooks by E.-L. Winnacker (Gene und Klone, Verlag Chemie, Weinheim, 1985) and by R.W. Old and S.B. Primrose (Principles of gene manipulation, Blackwell Scientific Publ., Oxford, 1985). The methods and techniques have in each case been updated to the most recent status e.g. in I.K. Setlow and A. Hollaender (Genetic engineering: principles and methods, Plenum Publ. Corp., N.Y.). Besides the specific publications, general working directions in the form of laboratory manuals have also been utilized in many instances [R.W. Davis et al., Advanced bacterial genetics: a manual for genetic engineering; T. Maniatis et al., Molecular cloning; T.J. Silhavy et al., Experiments with gene fusions, all from Cold Spring Harbor Lab., N.Y.; A Pühler and K.N. Timmis, Advanced molecular genetics, Springer, Berlin, 1984; D.M. Glover, DNA cloning: a practical approach, JRL Press, Oxford, Washington, D.C., 1985].

On August 28, 1986, the following microorganisms were deposited with the Deutsche Sammlung von Mikroorganismen [German Collection of Microorganisms] (DSM) in Göttingen, Federal Republic of Germany (deposit number):

E. coli HMS 174 (pT7-5)	(DMS 3829)
E. coli HMS 174 (pT7-6)	(DSM 3830)
E. coli JA 221 (pGSS33)	(DSM 3831)
E. coli LE 392 (pTJSS'035)	(DSM 3832)
E. coli HB101 (pVK101)	(DMS 3833)
E. coli SK1592 (pKT231)	(DSM 3834)
E. coli S17-1 (pKJS31)	(DSM 3835)
E. coli S17-1 (pKJS32RHΔS')	(DMS 3836)
E. coli S17-1 (pKJS(X)630)	(DSM 3837)
E. coli SBC107 (pRME1)	(DSM 3838)
E. coli K38 (pGT1-2/pTJS'X535)	(DSM 3839)
Alcaligenes eutrophus JMP134 (pJP4)	(DSM 3840)

Alcaligenes eutrophis JMP222 (DSM 3841)
Alcaligenes eutrophus JMP134:Tn5-2 (pJP4:Tn5-2) (DSM 3842)
Alcaligenes eutrophus JMP134:Tn5-4 (pJP4:Tn5-4) (DSM 3843)

Description of the Figures

Abbreviations:

Ap	-	ampicillin resistance
Km	-	kanamycin resistance
kb	-	kilobases
M	-	molecular weight marker
kD	-	kilodalton

Figures:

Fig. 1 - shows the origin and restriction map of the HindIII fragment cloned from pJP4, having a size of 21 kb (Example 1) and of the thus derived subfragments (Examples 3-7), as well as the expression of phenoxyacetic acid degradation in Alcaligenes eutrophus JMP222 by the corresponding plasmids with a wide host range (Example 14). The arrow marks the exact location of tfdA in the plasmid pJP4.

Fig. 2,3,4 - shows the plasmids derived from pKT231 containing tfdA or parts of tfdA (Examples 3-7). The thin lines denote the proportion stemming from the vector plasmid; the thick lines denote the insert stemming from pJP4.

Fig. 5,6,7a - show the plasmids derived from the T7 promoter plasmids pT7-5 and pT7-6 with tfdA-containing insert fragments (Examples 8-11). The thin lines denote the proportion stemming from the vector plasmid; the thick

lines denote the insert stemming from pJP4.

Fig. 7b - shows the insertion of the omega fragment in the plasmid pTJS'X535 (Example 11).

Fig. 8 - shows specifically radiolabelled proteins on the autoradiogram of a polyacrylamide gel, which proteins are expressed in high yield by the tfdA-containing plasmids with the aid of the T7 promoter (Example 15).
1: pTJSS'035; 2: pTJS'B435; 3: pTJS'X535;
4: pTJSS'036; 5: pTJS'B436; 6: pTJS'X536.
<u>a</u> denotes completely marked total cell protein, <u>b</u> denotes protein specifically marked after induction of the T7 promoter.

Fig. 10a/10b - shows the base sequence of the BamHI/SalI fragment having a length of 2058 bases, on which the gene tfdA is transcribed from the 5' end towards the 3' end. The arrows designate the beginning and end of the coding region of tfdA (Example 16).

Example 1: Preparation of Plasmid pVJH21

(a) Isolation of Plasmids pJP4 and pVK101

Alcaligenes eutrophus JMP134 is incubated in 250 ml of PYF medium (peptone, 3 g/l; yeast extract, 3 g/l; fructose, 2 g/l) for 16 hours at 30° C. The plasmid pJP4 is isolated from the centrifuged cells in accordance with the procedure from J. Bacteriol. 135 : 227 (1978). The plasmid pVK101 [V.C. Knauf et al., Plasmid 8 : 45 (1982)] is isolated from E. coli HB101 according to the generally known process of alkaline lysis by T. Maniatis et al. (Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982)

(b) Cloning of the 21-kilobase HindIII Fragment from pJP4

A reaction mixture consisting of 20 µl (500 ng) of pJP4, 2.5 µl of TAS buffer (0.33 M tris-acetate, 0.65 M potassium acetate, 0.1 M magnesium acetate, 5 mM dithiothreitol (DTT), 30 mM spermidine, pH 7.9) and 2.5 µl (1 unit) of a diluted HindIII solution is incubated at 37° C for 120 minutes. A further reaction mixture consisting of 50 µl (14 µg) of pVK101, 5.5 µl of TAS buffer and 1 µl (20 units) of undiluted HindIII solution is incubated at 37° C for 90 minutes. Then 1 µl (30 units) of calf intestinal alkaline phosphatase (DIP, Boehringer, Mannheim) is added and the mixture is incubated for another 60 minutes. Respectively 9 µl of each of the two reaction mixtures is separated on a 0.7% low-melting agarose gel by electrophoresis. The gel is subsequently stained for 15 minutes in an ethidium bromide solution (5 µg/ml) and the DNA bands are made visible in UV 300 nm.

The individual 20 kb band of pVK101 restriction, as well as the second-largest band (21 kb) of pJP4 restriction are excised from the gel, both bands are combined, and the DNA is isolated from the agarose according to the procedure by T. Maniatis et al. (Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982). The ethanol-precipitated DNA is taken up in 10 µl of ligation buffer (66 mM Tris-HCl, 6.6 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, pH 7.5), combined with 1 µl (0.1 unit) of diluted T4-DNA ligase, and incubated for 16 hours at 14° C (= ligation batch). Competent cells of E. coli S17-1 [Biotechnology 1 : 784 (1983)] are produced according to the method by T.J. Silhavy et al. (Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1984), and 0.2 ml of the thus-obtaiend competent cells are mixed with 5 µl of ligation batch, left for 40 minutes on ice, then heated for 3 minutes to 42° C (= transformation), subsequently diluted with 2 ml of LB medium according to T. Maniatis et al. (cf, above), and incubated for 60 minutes at 30° C (= phenotypic expression). Then portions of respectively 0.2 ml are spread out on LB agar plates containing 10 µl/ml of tetracycline. The plates are cultured at 37° C for 16 hours. Several tetracycline-resistant colonies are found.

(c) Identification of pVJH2

In accordance with the process of alkaline lysis, an instant preparation of the plasmid DNA is performed on the thus-obtained tetracycline-resistant colonies, as described by T. Maniatis et al. The aforedescribed process of treatment with alkaline phosphatase ensures that each of the tetracycline-resistant clones contains the desired insert. The recombinant plasmids are identified by restriction

enzyme digestion with EcoRI and subsequent electrophoretic separation of the DNA fragments in a 0.7% agarose gel according to T. Maniatis et al. (compare above). By comparing the sizes of the thus-obtained EcoRI fragments with the size relationships known from the literature with respect to EcoRI and HindIII for pJP4 and pVK101 [R.H. Don, J. Bacteriol. 161 : 466 (1985) and V.C. Knauf et al., Plasmid 8 :45 (1982)], an exact identification is provided of the insert DNA as well as of its orientation in the vector plasmid pVK101.

(d) Restriction Map of the HindIII Insert in pVJH21

A thus-obtained strain containing the plasmid pVJH21 is incubated in 400 ml of LB medium containing 20 μg/ml of tetracycline for 16 hours at 37° C. From the centrifuged cells, the plasmid DNA is isolated according to the method of alkaline lysis by T. Maniatis et al. (see above). The isolated plasmid DNA of pVJH21 is digested in various batches with the enzymes EcoRI, BamHI and SacI individually, as well as with the following nine combinations of several enzymes: EcoRI/BamHI (1); EcoRI/SacI (2); BamHI/SacI (3); HindIII/EcoRI (4); HindIII/BamHI (5); HindIII/SacI (6); HindIII/EcoRI/BamHI (7); HindIII/EcoRI/SacI (8); and HindIII/BamHI/SacI (9). The DNA fragments are separated in a 0.7% agarose gel according to T. Maniatis et al. (see above), and their sizes are determined by comparison with HindIII-cut lambda-DNA. The restriction map is drawn with the thus-obtained data together with the size relationships known for the plasmids pJP4 [R.H. Don, J. Bacteriol. 161 : 466 (1985)] and pVK101 [V.C. Knauf et al., Plasmid 8 :45 (1982)] with respect to EcoRI, BamHI and HindIII.

Example 2: Production of Plasmid pGJS3

(a) Isolation of Plasmids pVJH21 and pGSS33

The recombinant strain E. coli S17-1 containing the plasmid pVJH21 prepared in Example 1, and E. coli JA221 with the vector plasmid pGSS33 [G.S. Sharpe, Gene 29 : 93 (1984)] are incubated in respectively 300 ml of LB medium containing 20 μg/ml of tetracycline for 16 hours at 37° C. The two plasmids are isolated from the centrifuged cells in accordance with the method of alkaline lysis by T. Maniatis et al. (see above).

(b) Cloning of SacI Fragments from pVJH21

A reaction mixture consisting of 10 μl (5 μg) of pVJH21, 10 μl (1 μg) of pGSS33, 4 μl of TAS buffer, 16 μl of water, and 0.5 μl (10 units) of SacI is incubated at 37° C for 90 minutes. The restriction enzyme is subsequently inactivated by heating to 68° C for 15 minutes. The DNA is precipitated with ethanol according to T. Maniatis et al. (see above), and the dried precipitate is taken up in 40 μl of ligation buffer. After addition of 1 μl (1 unit) of T4-DNA ligase, the ligation batch is incubated for 16 hours at 14° C. E. coli LE392 [N.E. Murray et al., Mol. Gen. Genet. 150 : 53 (1977)] is rendered competent for DNA reception according to the method by T.J. Silhavy et al. (Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1984); 0.2 ml of competent cells is combined with 5 μl of ligation batch, left on ice for 40 mintues, and transformed by 3 minutes of heating to 42° C. After dilution with 2 ml of LB medium and incubation at 30° C, portions of 50 μl to 200 μl are spread on LB agar plates containing 20 μg/ml of chloramphenicol. The plates are incubated for 16 hours at 37° C.

(c) Identification of Recombinant Plasmids

The thus-obtained chloramphenicol-resistant clones are spread parallel on two LB agar plates, one of which contains 25 µg/ml of streptomycin, the other of which contains 20 µg/ml of chloramphenicol. The plasmid DNA is isolated from clones that prove streptomycin-sensitive in this test with the aid of the instant method of alkaline lysis according to T. Maniatis et al. (see above). The size of the inserts contained in vector pVK101 is determined by restriction of the isolated plasmids with SacI, electrophoretic separation of the DNA fragments on a 0.7% agarose gel, and comparison of the bands visible in UV 300 nm with known fragments of a HindIII restriction of lambda phage DNA. The plasmid pGJS3 is a recombinant plasmid from pGSS33 and a DNA fragment having a size of 3 kilobases. Since the plasmid pVK101 per se has no scission site for SacI, the cloned DNA fragment clearly stems from the HindIII fragment, having a size of 21 kilobases, of pJP4.

Example 3: Preparation of Plasmids pKJS31 and pKJS32

(a) Isolation of Plasmids pGJS3 and pKT231

The strain E. coli LE392 produced in Example 2, containing the recombinant plasmid pGJS3, is cultured in 400 ml of LB medium with an addition of 20 µg/ml of chloramphenicol for 16 hours at 37° C. E. coli SK1592 with the vector plasmid pKT231 [M. Bagdasarian et al., Current Topics in Microbiology and Immunology, 96 : 47 (1982)] is incubated in 400 ml of LB medium with an addition of 50 µg/ml of kanamycin under the same conditions. The two plasmids are isolated from the centrifuged cells according to the process of alkaline lysis by T. Maniatis et al. (see above).

(b) Transcloning of the SacI Fragment from pGJS3 into pKT231

A reaction mixture consisting of 40 µl (400 ng) of pGJS3, 4 µl (100 ng) of pKT231, 6 µl of TAS buffer, 9 µl of water, and 1 µl (1 unit) of dilute SacI solution is incubated at 37° C for 120 minutes. The restriction enzymes are inactivated by subsequent heating of the reaction mixture to 68° C for 15 minutes. The batch is combined with 39 µl of water, 10 µl of 10-fold concentrated ligation buffer, and 1 µl (0.1 unit) of a dilute T4-DNA ligase, and the ligation batch is incubated for 16 hours at 14° C. An amount of 0.2 ml of competent cells of E. coli LE392, prepared according to the process by T.J. Silhavy et al. (Experiments with Gene Fusion, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1984) is mixed with 10 µl of the ligation batch, left on ice for 40 minutes, heated for 3 minutes to 42° C, mixed with 2 ml of LB medium, and incubated at 37° C for 60 minutes. Portions of 50 µl to 200 µl are spread on LB agar plates containing 50 µg/ml of kanamycin. The plates are incubated at 37° C for 16 hours.

(c) Identification of Recombinant Plasmids

The thus-obtained kanamycin-resistant clones are spread in parallel on three different LB agar plates, one of which contains 25 µg/ml of streptomycin, the second containing 20 µg/ml of chloramphenicol, and the third plate containing 50 µg/ml of kanamycin. Clones that turn out in this test to be sensitive to streptomycin and chloramphenicol, but resistant to kanamycin contain, in the normal case, a recombinant plasmid from the vector pKT231 (kanamycin resistance) and from the 3-kilobase SacI fragment from pGJS3. The size of the inserted DNA is determined by isolating

the plasmid DNA according to the conventional instant method by T. Maniatis et al. (see above), restriction of the DNA with SacI and subsequent analysis of the fragments by electrophoresis in a 0.7% agarose gel. Plasmids having differing orientation of the insert in relation to the vector plasmid are identified by a restriction analysis with EcoRI carried out in the same way. The plasmid pKJS32 constitutes that form of the two possibilities wherein the EcoRI site of the insert is located closest to the EcoRI site of the vector proportion. This plasmid yields two EcoRI fragments of a size of 0.9 and 15 kilobases. The plasmid pKJS31 represents the construction with the opposite orientation of the insert, yielding two EcoRI fragments of the sizes 2.3 and 13.5 kilobases.

Example 4: Production of Plasmid pKJSB330

(a) Isolation of Plasmid pKJS31

The clone of E. coli LE392 obtained in Example 3 and containing the recombined plasmid pKJS31 is incubated in 400 ml of LB medium with an addition of 50 µg/ml of kanamycin at 37° C for 16 hours, and from the centrifuged cells the plasmid DNA is isolated by means of alkaline lysis according to T. Maniatis et al. (see above).

(b) Deletion of Smaller BamHI Fragment from pKJS31

A reaction mixture consisting of 10 µl (500 ng) of pKJS31, 7 µl of water, 2 µl of TAS buffer and 1 µl (1 unit) of a diluted BamHI solution is incubated for 60 minutes at 37° C, and subsequently the enzymatic reaction is stopped by heating to 68° C for

15 minutes. The restriction batch is then combined with 50 µl of water, 8 µl of 10-fold concentrated ligation buffer, and 2 µl (2 units) of T4-DNA ligase and incubated for 16 hours at 14° C. A mixture is prepared from 0.2 ml of competent cells of E. coli S17-1, produced according to the conventional method by T.J. Silhavy et al. (Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1984), and 10 µl of the ligation batch, left on ice for 40 minutes, heated to 42° C for 3 minutes, mixed with 2 ml of LB medium, and incubated for 60 minutes at 37° C. Portions of 50 µl to 200 µl are spread on LB agar plates containing 50 µg/ml of kanamycin. The plates are incubated for 16 hours at 37° C.

(c) Identification of the Shortened Plasmids

The plasmid DNA is isolated from the thus-obtained kanamycin-resistant clones by means of the known instant method of alkaline lysis by T. Maniatis et al. (see above). By restriction of the plasmids with BglII and and electrophoretic separation of the DNA fragments in a 0.7% agarose gel, plasmids are identified which have lost the small BamHI fragment from pKJS31. These plasmids possess only a single BglII scission site and therefore yield a BglII fragment having a size of 13.2 kilobases. They can thus be clearly differentiated from their origin plasmid pKJS31 and are denoted as pKJSB330.

<u>Example 5: Production of Plasmid pKJS32RHΔS'</u>

(a) Isolation of Plasmids pKJS32 and pRME1

The clone of E. coli LE392 obtained in Example 3 and containing the recombined plasmid pKJS32, as well as E. coli SBC107 (pRME1) are cultured in respectively 400 ml of LB medium with an addition of

50 μg/ml of kanamycin at 37° C for 16 hours. The plasmids are isolated from the centrifuged cells by alkaline lysis according to T. Maniatis et al. (see above).

(b) Deletion of a HindIII/SalI Fragment from pKJS32 and Subsequent Insertion of a HindIII/SalI Fragment from pRME1

A reaction mixture consisting of 10 μl (500 ng) of pKJS32, 10 μl (1 μg) of pRME1, 15 μl of water, 4 μl of TAS buffer, 0.5 μl (1 unit) of dilute HindIII solution, and 0.5 μl (1 unit) of dilute SalI solution is incubated for 120 minutes at 37° C, and the enzymatic reaction is stopped by heating to 68° C for 15 minutes. Then 10 μl of the restriction batch is combined with 25 μl of water, 4 μl of 10-fold concentrated ligation buffer and 1 μl (1 unit) of T4-DNA ligase and the mixture is incubated for 16 horus at 14° C. Cells of E. coli S17-1 rendered competent according to a conventional process by T.J. Silhavy et al. (Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1984) are mixed with 10 μl of the ligation batch, left on ice for 40 minutes, heated for 3 minutes to 42°C, and after mixing with 2 ml of LB medium, spread on LB agar plates containing 50 μg/ml of kanamycin. The plates are incubated for 16 hours at 37°C.

(c) Identification of the Recombinant Plasmids

The plasmid DNA is isolated from the thus-produced kanamycin-resistant clones by the conventional instant method of alkaline lysis according to T. Maniatis et al. (see above). By restriction with BamHI and electrophoretic separation of the resultant DNA fragments, plasmids are identified wherein, starting from pKJS32, the smaller DNA segment between the HindIII cutting site located on the vector portion in the middle of the kanamycin resistance gene and the SalI cutting site located on the

insert has been removed and replaced by that HindIII/SalI fragment from pRME1 carrying the 3' end of the coding region of the kanamycin resistance gene. These plasmids yield two BamHI fragments having the lengths of 13.3 and 2.0 kilobases and thus clearly differ from the starting products and other possible byproducts of the ligation. They represent structures wherein a 0.2-kilobase DNA segment of the 3.0-kilobase insert of pKJS32 has been removed and wherein a part of the kanamycin resistance gene has been exchanged against another gene fragment of the same genetic origin. These plasmids are called pKJS32RHΔS'.

Example 6: Preparation of Plasmid pKJEΔB130

(a) Isolation of Plasmids pKT231 and pKJS32

The isolation of pKT231 was described in Example 3, that of pKJS32 in Example 5.

(b) Cloning of the Smaller EcoRI/BamHI Fragment from pKJS32 in pKT231

Two reaction mixtures, one consisting of 10 µl (500 ng) of pKJS32, 5 µl of water, 2 µl of TAS buffer, and respectively 1 µl (1 unit) of dilute solutions of the enzymes EcoRI, BamHI and PstI, the other consisting of 10 µl (250 ng) of pKT231, 6 µl of water, 2 µl of TAS buffer, and respectively 1 µl (1 unit) of dilute solutions of the enzymes EcoRI and BamHI, are incubated for 120 minutes at 37° C and, to stop the reaction, heated for 15 minutes to 68° C. Subsequently respectively 10 µl of the two restriction batches are mixed, combined with 50 µl of water, 8 µl of 10-fold concentrated ligation buffer and 2 µl (2 units) of T4-DNA ligase, and incubated for 16 hours at 14° C. A mixture is prepared from 0.2 ml of competent cells of E. coli LE392, produced according to the process known in

the literature by T.J. Silhavy et al. (Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1984), and 10 µl of the ligation batch, left on ice for 40 minutes, heated to 42° C for 3 minutes, mixed with 2 ml of LB medium, and incubated at 37° C for 60 minutes. Portions of 50 µl to 200 µl are spread on LB agar plates containing 50 µg/ml of kanamycin. The plates are incubated at 37° C for 16 hours.

(c) Identification of Recombinant Plasmids

The thus-obtained kanamycin-resistant clones are spread in parallel on two LB agar plates, one of which contains 25 µg/ml of streptomycin, the other 50 µg/ml of kanamycin. The plasmid DNA is isolated from clones proving to be streptomycin-resistant in this test, in accordance with the instant method by T. Maniatis et al. (see above). Restriction of the DNA with XhoI and subsequent electrophoretic separation of the DNA fragments in a 0.7% agarose gel permit identification of recombinant plasmids yielding two XhoI fragments having the sizes of 3.0 and 9.7 kilobases. When cutting the two plasmids simultaneously with EcoRI and BamHI, electrophoretic analysis yields two fragments having the sizes of 1.5 and 11.2 kilobases. The thus-identified plasmids are recombinants from the large EcoRI/BamHI fragment of pKT231 and the 1.5-kilobase EcoRI/BamHI fragment stemming from the insert DNA of plasmid pKJS32. They are called pKJEΔB130.

Example 7: Production of Plasmid pKJS(X)630

(a) Isolation of Plasmid pKJSB330

The clone of E. coli S17-1, obtained in Example 4 and containing the recombinant plasmid pKJS330, is cultured in 400 ml of LB medium with an addition of 50 µg/ml of kanamycin at 37° C for 16 hours, and the plasmid DNA is isolated from the centrifuged cells by alkaline lysis according to T. Maniatis et al. (see above).

(b) Removal of a BamHI/XbaI Fragment from Plasmid pKJSB330

A reaction mixture consisting of 20 µl (1 µg) of pKJSB330, 15 µl of water, 4 µl of TAS buffer, 0.5 µl (2 units) of dilute XbaI solution, and 0.5 µl (2 units) of dilute BamHI solution is incubated at 37° C for 120 minutes. Then 20 µl of buffered phenol according to T. Maniatis et al. (see above) is added, the batch is mixed, then again blended after adding 20 µl of chloroform : isoamyl alcohol (24:1), and removed by centrifuging. The top aqueous phase is taken off and mixed with 120 µl of cold ethanol of a temperature of -20° C. The mixture is stored at -20° C for 30 minutes and then removed by centrifuging. The precipitated DNA is washed with cold 70% strength ethanol, dried under vacuum, and taken up in 20 µl of Klenow reaction solution [20 mM Tris-HCl, pH 8.0, 7 mM $MgCl_2$, 10 U/ml DNA polymerase I (= Klenow enzyme)]. After preliminary incubation at 37° C for 5 minutes, 2 µl of a solution of all four deoxynucleotide triphosphates (0.125 mM ATP, 0.125 mM CTP, 0.125 mM GTP, 0.125 mM TTP, Pharmacia) is added and incubation is continued for another 5 minutes. Then the batch is mixed with 80 µl of ligation buffer containing 25 U/ml of T4-DNA ligase and left at room temperature for 6 hours.

Then the mixture is incubated at 14° C for 16 hours, 0.2 ml of a suspension of competent cells of E. coli S17-1 prepared according to the method by T.J. Silhavy et al. (Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1984) is mixed with 10 µl of the ligation batch, transformed, after 40 minutes of incubation on ice, by heat treatment (3 minutes, 42° C), mixed with 2 ml of LB medium, and incubated at 37° C for 60 minutes. Then portions of 50 to 200 µl of the cell suspension are spread on LB agar plates containing 50 µg/ml of kanamycin. The plates are incubated at 37° C for 16 hours.

(c) Identification of the Shortened Plasmids

From the thus-obtained kanamycin-resistant clones, the plasmid DNA is isolated by the conventional instant method of alkaline lysis according to T. Maniatis et al. (see above). By restriction of the plasmids with SmaI and subsequent electrophoretic separation of the DNA fragments in a 0.7% agarose gel, plasmids are identified which have lost the DNA segment between the BamHI scission site and the XbaI scission site on the insert of pKJSB330. These plasmids yield, in the restriction analysis, two SmaI fragments having a length of 2.6 and 9.9 kilobases and are called pKJS(X)630. They are thus to be clearly distinguished from the starting product pKJSB330.

Example 8: Production of Plasmids pTJSS'035 and pTJSS'036

(a) Isolation of Plasmids pT7-5, pT7-6 and pKSJ32

Two strains of E. coli HMS174, one of which contains the plasmid pT7-5, the other the plasmid pT7-6, are cultured in respectively 400 ml of LB medium according to T. Maniatis et al. (see above) with an addition of 50 µg/ml of ampicillin at 37° C for 16 hours. From the centrifuged cells, the plasmid DNA is isolated by the known method of alkaline lysis by T. Maniatis. Isolation of pKJS32 is described in Example 5.

(b) Cloning of the SacI/SalI Fragments from pKJS32 in pT7-5 and pT7-6

A reaction mixture (A), consisting of 20 µl (1 µg) of pKJS32, 15 µl of water, 4 µl of TAS buffer, and respectively 0.5 µl (2 units) of the enzymes SacI and SalI, is incubated for 120 minutes at 37° C. Two further reaction mixtures, consisting either of 2 µl (1 µg) of pT7-5 (B) or 2 µl (1 µg) of pT7-6 (C), 15 µl of water, 2 µl of TAS buffer, and respectively 0.5 µl (2 units) of the enzymes SacI and SalI, are incubated under identical conditions. All three reactions are stopped by heating to 68° C for 15 minutes. Respectively 13 µl of the restriction batch (A) (pKJS32) is mixed with 7 µl of the restriction batch (B) (pT7-5) and, respectively, 7 µl of the restriction batch (C) (pT7-6). The two mixtures are combined with respectively 50 µl of water, 8 µl of 10-fold concentrated ligation buffer, and 2 µl (2 units) of T4-DNA ligase. Both ligation batches are incubated at 14° C for 16 hours. Respectively 0.2 ml of competent cells of E. coli LE392, prepared according to the procedure by T.J. Silhavy et al. (Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, New

York, 1984), is mixed with respectively 10 µl of the ligation batches, left on ice for 40 minutes, heated to 42° C for 3 minutes, mixed with 2 ml of LB medium, and incubated at 37° C for 60 minutes. Portions of 50 µl to 200 µl are spread on LB agar plates containing 50 µg/ml of ampicillin. The plates are incubated at 37° C for 16 hours.

(c) Identification of Recombinant Plasmids

The plasmid DNA is isolated from the resultant ampicillin-resistant clones by the known instant method of alkaline lysis according to T. Maniatis et al. (see above). By restriction of the plasmids with BglII and BamHI, as well as by dual restriction with BamHI and HindIII and subsequent electrophoretic separation of the fragments in a 0.7% agarose gel, plasmids are identified consisting of one of the two vector plasmids pT7-5 or pT7-6 and the small SacI/SalI fragment of pKJS32. These recombinant plasmids yield in this case, with pT7-5 being the starting material, two BglII fragments having a size of 3.0 kilobases and 2.2 kilobases, a BamHI fragment of a size of 5.2 kilobases, and two BamHI/HindIII fragments having the sizes of 3.2 and 2.0 kilobases, and they are called pTJSS'035. In case of pT7-6 as the starting material, two BglII fragments are obtained of a size of 2.8 kilobases and 2.2 kilobases, a BamHI fragment of a size of 5.0 kilobases, and two BamHI/HindIII fragments of the sizes 3.0 kilobases and 2.0 kilobases; they are called pTJSS'036. The recombinant plasmids thus differ clearly from their starting products pKJS32 and pT7-5 or pT7-6.

Example 9: Preparation of Plasmids pTJS'B435 and pTJS'B436 :

(a) Isolation of Plasmids pT7-5, pT7-6 and pKJSB330

Isolation of pT7-5 and pT7-6 has been described in Example 8; the isolation of pKJSB330 has been disclosed in Exampel 7.

(b) Cloning of the Short SalI/BamHI Fragment from pKJSB330 in pT7-5 and pT7-6

A reaction mixture (A), consisting of 40 µl (2 µg) of pKJSB330, 5 µl of TAS buffer, and respectively 2.5 µl (4 units) of dilute solutions of the enzymes SalI and BamHI, is incubated at 37° C for 120 minutes. Two further reaction mixtures, consisting of 2 µl (1 µg) of pT7-5 (B) and, respectively, 2 µl (1 µg) of pT7-6 (C), 20 µl of water, 3 µl of TAS buffer, and respectively 2.5 µl (4 units) of dilute solutions of the enzymes SalI and BamHI, are incubated under identical conditions. The reactions are stopped by heating to 68° C for 15 minutes. The entire restriction batch (A) (pKJSB330) is separated by electrophoresis in an agarose gel [0.7% of agarose in TBE buffer according to T. Maniatis et al. (see above) containing 0.5 µg/ml of ethidium bromide, according to T. Maniatis et al. (see above)]. Of the two DNA bands visible under UV light (300 nm), the shorter band of 2.0 kilobases is isolated from the gel by the method of DEAE membrane elution in the following way: A suitably dimensioned section of a DEAE membrane (Schleicher and Schuell, S & S NA-45) is placed into a cut 1-2 mm below the band to be eluted. The electrophoresis is continued until the respective

band has been entirely absorbed by the membrane. The membrane is then removed from the gel and washed for 10 minutes with 10 ml of NET buffer (150 mM NaCl, 0.1 mM EDTA, 20 mM Tris-HCl, pH 8.0). The washed membrane is incubated in 150 µl of HNET buffer (1 M NaCl, 0.1 mM EDTA, 20 mM Tris-HCl, pH 8.0) for 30 minutes at 68° C. The solution is removed and the membrane incubated another 10 minutes with 50 µl of HNET buffer. The combined elution solutions (200 µl) are diluted with 200 µl of water, mixed with 800 µl of cold (-20° C) ethanol, and stored at -20° C for 30 minutes. The precipitated DNA is centrifuged and the precipitate taken up in 90 µl of water. After adding 10 µl of 3-molar sodium acetate solution according to T. Maniatis et al. (see above), the mixture is reprecipitated with 200 µl of cold ethanol (30 minutes, -20° C), centrifuged, the precipitate washed with cold 70% ethanol, dried under vacuum, and taken up in 80 µl of ligation buffer. Of this mixture, respectively 40 µl is combined with 3 µl (100 ng) of the restriction batch (B) (pT7-5) and, respectively, 3 µl (100 ng) of the restriction batch (C) (pT7-6), and 2 µl (1 unit) of T4-DNA ligase, and incubated at 14° C for 16 hours. An amount of 0.2 ml of competent cells of E. coli LE 392, prepared according to the method by T.J. Silhavy et al. (Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1984), is mixed with 15 µl of the ligation batch, left on ice for 40 minutes, heated to 42° C for 3 minutes, mixed with 2 ml of LB medium, and incubated at 37° C for 60 minutes. Portions of 50 µl to 200 µl are spread on LB agar plates containing 50 µg/ml of ampicillin. The plates are incubated at 37° C for 16 hours.

(c) Identification of Recombinant Plasmids

The DNA is isolated from the resultant ampicillin-resistant clones according to the known instant method of alkaline lysis by T. Maniatis et al. (see above). Restriction of the plasmids with EcoRI and subsequent electrophoretic separation of the fragments in a 0.7% agarose gel afford identification of plasmids containing the short SalI/BamHI fragment from pKJSB330 in one of the two vector plasmids. Recombinant plasmids with pT7-5 as the starting product yield two EcoRI fragments of 3.0 kilobases and 1.5 kilobases in size and are called pTJS'B435; those with pT7-6 as the starting material yield two EcoRI fragments of 2.8 kilobases and 1.5 kilobases in size and are called pTJS'B436.

Example 10: Production of Plasmids pTJS'X535 and pTJS'X536

(a) Isolation of Plasmids pT7-5, pT7-6 and pTJSS'035

E. coli LE392 containing the plasmid pTJSS'035 produced in Example 8 is cultured in 400 ml of LB medium with an addition of 50 µg/ml of ampicillin at 37° C for 16 hours. From the centrifuged cells, the plasmid DNA is isolated according to T. Maniatis et al. (see above). Isolation of pT7-5 and pT7-6 has been set forth in Example 8.

(b) Cloning of the Short SalI/XbaI Fragment from pTJSS'035 in pT7-5 and pT7-6

A reaction mixture (A), consisting of 6 µl (3 µg) of pTJSS'035, 15 µl of water, 3 µl of TAS buffer, and respectively 2 µl (5 units) of dilute solutions of the enzymes XbaI, SalI and BamHI, is incubated

at 37° C for 120 minutes. Two further reaction mixtures, consisting of either 2 µl (1 µg) of pT7-5 (B) or 2 µl (1 µg) of pT7-6 (C), 23 µl of water, 3 µl of TAS buffer, and respectively 1 µl (2.5 units) of dilute solutions of the enzymes XbaI and SalI, are incubated in the same way. The reactions are stopped by heating to 68° C for 15 minutes. In two different ligation batches, respectively 5 µl (500 ng) of the restriction batch (A) (pTJSS'035) is mixed with either 15 µl of restriction batch (B) (pT7-5) or 15 µl of restriction batch (C) (pT7-6), 50 µl of water, 8 µl of ligation buffer, and 2 µl (2 units) of T4-DNA ligase, and incubated at 14° C for 16 hours. A mixture is prepared from 0.2 ml of competent cells of E. coli LE392, prepared according to the method by T.J. Silhavy et al. (Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1984) and 15 µl of the ligation batch, left on ice for 40 minutes, heated to 42° C for 3 minutes, mixed with 2 ml of LB medium, and incubated at 37° C for 60 minutes. Portions of 50 µl to 200 µl are spread on LB agar plates containing 50 µg/ml of ampicillin. The plates are incubated at 37° C for 16 hours.

(c) Identification of Recombinant Plasmids

From the thus-obtained ampicillin-resistant clones, the plasmid DNA is isolated according to T. Maniatis et al. (see above). By restriction with the enzymes SmaI and EcoRI, as well as by dual restriction with EcoRI and SalI, plasmids are identified which contain the short, 1.4-kilobase SalI/XbaI fragment from pTJSS'035 in one of the two vector plasmids pT7-5 and, respectively, pT7-6. Recombinant plasmids with pT7-5 as the starting material yield two EcoRI fragments 3.0 and 0.8 kilobases in size, two SmaI fragments of the sizes 2.4 and 1.4 kilobases, and three EcoRI/SalI fragments of the sizes

2.4, 0.8 and 0.6 kilobases; they are called pTJS'X535. Recombinant plasmids with pT7-6 as the starting material yield two EcoRI fragments of the sizes of 2.8 and 0.8 kilobases, two SmaI fragments having the sizes of 2.2 and 1.4 kilobases, and three EcoRI/SalI fragments having the sizes 2.2, 0.8 and 0.6 kilobses; they are called pTJS'X536. The recombinant plasmids thus differ unequivocally from their starting materials.

Example 11: Preparation of Plasmid pTJS'X535omega

(a) Isolation of Plasmids pTJS'X535 and pDOC37

Two E. coli strains LE392, one of which contains the plasmid pTJS'X535 prepared in Example 10, the other the plasmid pDOC37, are cultured in respectively 400 ml of LB medium with an addition of 50 µg/ml of ampicillin at 37° C for 16 hours. The plasmid DNA is isolated from the centrifuged cells in accordance with T. Maniatis et al. (see above). The plasmid pDOC37 contains the omega fragment between two EcoRI restriction sites. Instead of pDOC37, it is likewise possible to utilize, as a source for the omega fragment, the plasmid pHP45omega described by Prentki and Krisch, 1984, in Gene 29 : 103.

(b) Cloning of the Omega Fragment from pDOC37 into the BglII Site of pTJS'X535

Two reaction mixtures, one of which consists of 2 µl (1 µg) of pTJS'X535, 14 µl of water, 2 µl of TAS buffer, and 2 µl (3 units) of BglII, the other of which consists of 2 µl (2 µg) of pDOC37, 14 µl of water, 2 µl of TAS buffer, and 2 µl (4 units) of a dilute BamHI solution, are incubated at 37° C for 120 minutes. The reactions are stopped by heating to 68° C for 15 minutes. Respectively 10 µl of both restriction batches are combined and

mixed with 80 µl of ligation buffer and 1 µl (1 unit) of T4-DNA ligase. The ligation batch is incubated at 14° C for 16 hours. An amount of 0.2 ml of competent cells of E. coli LE 392, prepared according to T.J. Silhavy et al. (Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1984), is mixed with 10 µl of the ligation batch, left on ice for 40 minutes, heated to 42° C for 3 minutes, mixed with 2 ml of LB medium, and incubated at 37° C for 60 minutes. Portions of 50 µl to 200 µl are spread on LB agar plates containing 50 µg/ml of ampicillin and 80 µg/ml of spectinomycin. The plates are incubated at 37° C for 16 hours. By selection with spectinomycin, it is ensured that all growing clones contain a plasmid with the omega fragment since the latter contains the gene for expression of spectinomycin resistance.

(c) Identification of Recombinant Plasmids

The plasmid DNA is isolated from the thus-obtained ampicillin- and spectinomycin-resistant clones according to T. Maniatis et al. (see above). By restriction with HindIII, the recombinant plasmids are identified. If three HindIII fragments are thus obtained having the sizes of 0.6, 2.0 and 3.2 kilobases, these involve derivatives of pTJS'X535 which have the omega fragment built in at the previous BglII scission site. By ligation of the compatible overlapping ends of the BglII and BamHI fragments, the restriction sites BglII and BamHI at the linkage point are lost. These recombinant plasmids are called pTJS'X535omega.

Example 12: Production of Phages MJSS'030 and MJSS'031

(a) Isolation of Plasmid pKJS32 and the Double-Strand Forms of Phages M13tg130 and M13tg131

The strain E. coli JM101 [J. Messing et al., Nucl. Acids Res. 9 : 309 (1981)] as well as double-strand DNA of the vectors M13tg130 and M13tg131 [M.P. Kieny et al., Gene 26 : 91 (1983)] can be obtained from Amersham Buchler GmbH & Co. KG, Braunschweig. Double-strand DNA can also be obtained according to the following method: 0.2 ml of competent cells of E. coli JM101, produced according to T.J. Silhavy et al. (Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1984) are transformed with single-strand or double-strand DNA of the M13 vectors according to a process known from the literature, mixed with 3 ml of melted (45° C) H-Top agar (10 g/l of tryptone, 8 g/l of NaCl, 8 g/l of agar), and poured on H-agar plates (10 g/l of tryptone, 8 g/l of NaCl, 12 g/l of agar). The cooled plates are incubated at 37° C for 16 hours. 2 ml of TY medium (16 g/l of tryptone, 10 g/l of yeast extract, 5 g/l of NaCl) is inoculated with 20 µl of an overnight culture (16 hours, 37° C) of E. coli JM101 in TY medium and a single plaque of the H-agar plate. After 16 hours of growth at 37° C, the phage-infected cells, together with a fresh overnight culture of E. coli JM101, are transferred by inoculation into 400 ml of TY medium and cultured for 16 hours at 37° C. From the centrifuged cells, the double-strand DNA is isolated according to T. Maniatis et al. (see above). Isolation of plasmid pKJS32 has been described in Example 5.

(b) Cloning of the SacI/SalI Fragment from pKJS32 into M13tg130 and M13tg131

A reaction mixture (A), consisting of 30 µl (1.5 µg) of pKJS32, 20 µl of water, 6 µl of TAS buffer, and respectively 2 µl (4 units) of dilute solutions of the enzymes SacI and SalI, is incubated at 37° C for 120 minutes. Two further reaction mixtures, consisting of 10 µl (300 ng) of double-strand DNA of M13tg130 (B) or M13tg131 (C), 25 µl of water, 4 µl of TAS buffer, and respectively 0.5 µl (1 unit) of dilute solutions of the enzymes SacI and SalI, are incubated in the same way and heated, for stopping the reaction, to 68° C for 15 minutes. The entire restriction batch (A) is separated by electrophoresis in an agarose gel [0.7% agarose, 0.5 µg/ml of ethidium bromide in TBE buffer according to T. Maniatis et al. (see above)]. Of the two bands visible under UV light, the smaller SacI/SalI band of 2.8 kilobases is isolated by means of a DEAE membrane from the gel in the same way as has been described for the 2.0-kilobase band in Example 9. The DNA is finally taken up in 80 µl of water; of this amount, respectively 40 µl is combined with 20 µl of the restriction batches (A) (M13tg130) and, respectively, (B) (M13tg131) and 40 µl of water, mixed with 100 µl of buffered phenol/chloroform according to T. Maniatis et al. (see above), and centrifuged. The top aqueous phases are removed and, after adding 10 µl of 3-molar sodium acetate solution according to T. Maniatis et al. (see above), mixed with respectively 200 µl of cold (-20° C) ethanol. The mixtures are left at -20° C for 30 minutes, subsequently centrifuged, the precipitates are washed with cold 70% ethanol, dried under vacuum, and taken up in 40 µl of ligation buffer. The two batches are combined with respectively 1 µl (1 unit) of T4-DNA ligase and incubated at 14° C for 16 hours. Respectively 0.2 ml of

competent cells of E. coli JM101, prepared according to T.J. Silhavy et al. (Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1984), is mixed with 10 µl of the ligation batches, left on ice for 40 minutes, heated to 42° C for 3 minutes, and combined with respectively 3 ml of melted (45° C) H-Top agar. The mixtures are combined with respectively 40 µl of IPTG (100 mM) and 40 µl of Xgal (2% in dimethylformamide) and poured on H-agar plates. The cooled plates are incubated at 37° C for 16 hours.

(c) Identification of Recombinant Phages

Among the thus-obtained plaques, those stem from recombinant phages which do not exhibit a blue coloration. For further identification, the double-strand and single-strand DNA is isolated from the colorless plaques according to methods known from the literature, compiled in "M13 Cloning and Sequencing Handbook" by Amersham. Restriction of the double-strand DNA with BglII and subsequent electrophoretic separation of the DNA fragments identify derivatives of M13gt130 and, respectively, M13tg131 which contain the short SacI/SalI fragment from pKJS32. Recombinant phages with M13tg130 as the vector yield three BglII fragments of the sizes 0.7, 2.2 and 7.1 kilobases and are denoted as MJSS'030; those with M13tg131 yield four BglII fragments of the sizes 0.6, 0.7, 2.2 and 6.6 kilobases and are called MJSS'031. They differ accordingly clearly from their starting products.

Example 13: Production of the tfdA Mutants Alcaligenes eutrophus JMP134:Tn5-2 and JMP134:Tn5-4

(a) Transposon Mutagenesis of Alcaligenes eutrophus JMP134

10 ml of PYE mdium (3 g/l of peptone, 3 g/l of yeast extract) is inoculated with 0.5 ml of an overnight culture (16 hours, 30° C) of Alcaligenes eutrophus JMP134 [R.H. Don et al., J. Bacteriol. 145 : 681 (1981)] and shaken for 8 hours at 30° C. 10 ml of LB medium according to T. Maniatis et al. (see above) is inoculated with 0.1 ml of an overnight culture of E. coli S17-1 [Biotechnology 1 ; 784 (1983)] containing the plasmid pSUP2021, and shaken for 5 hours at 37° C. Subsequently, the optical density at a wavelength of 600 nm is determined in a photometer for both cultures. The cultures are mixed in a proportion of 1 : 1 with respect to their optical density and 1.5 ml of the mixture is distributed on a PYE agar plate [R.H. Don et al., J. Bacteriol. 145 : 681 (1981)]. The plate is incubated for 16 hours at 30° C. Subsequently the bacteria are rinsed off the plate with 1.5 ml of sterile 0.7% NaCl solution and the bacterial suspension is diluted in two steps with 0.7% NaCl solution respectively 1 : 10 (0.1 ml + 0.9 ml). Of each dilution, portions of 50 µl, 100 µl, 200 µl and 400 µl are spread on minimal agar plates (1.6 g/l of dipotassium hydrogen phosphate, 0.4 g/l of potassium dihydrogen phosphate, 1 g/l of ammonium sulfate, 0.05 g/l of magnesium sulfat x 7 water, 0.01 g/l of iron(II) sulfate x 7 water, 15 g/l of agar), these plates additionally containing 2 g/l of fructose and 380 µg/ml of kanamycin. The plates are incubated at 30° C for 3 to 7 days.

(b) Testing the Transposon-Containing Strains for 2,4-D Degradation

The thus-obtained kanamycin-resistant clones represent progeny of the strain JMP134 which have integrated the transposon Tn5 in a stable fashion in their genome. They are spread in parallel on two minimal agar plates, one of which contains 1 mM of 2,4-dichlorophenoxyacetic acid, sodium salt (2,4-D), the other of which contains 2 g/l of fructose and 380 µg/ml of kanamycin. The plates are incubated for 3 days at 30° C. Strains exhibiting mutation in one of the genes responsible for 2,4-D degradation cannot utilize 2,4-D as a growth substrate (2,4-D-negative). They occur at a frequency of 0.1 - 1%, based on the entirety of transposon-containing, kanamycin-resistant clones.

(c) Identification of the Gene Defect of the 2,4-D-Negative Mutants

The thus-obtained 2,4-D-negative mutants are spread in parallel on two minimal agar plates, one of which contains 2 mM of 3-chlorobenzoic acid, sodium salt (3-CB), the other 2 g/l of fructose and 380 µg/ml of kanamycin. The plates are incubated for 3 days at 30° C. 2,4-D-negative transposon mutants of the strain JMP134 exhibiting mutation in the genes tfdC, tfdD and tfdE of 2,4-D degradation cannot utilize 3-CB as a growth substrate (3-CB-negative), as could be shown by Don et al. [J. Bacteriol. 161 : 85 (1985)]. Mutants in the genes tfdA and tfdB, in contrast thereto, can grow on 3-CB as the sole carbon source (3-CB-positive). Transposon mutants proving to be 3-CB-positive in this test are accordingly further investigated by means of an enzyme test for defects in the genes tfdA or tfdB. For this purpose, the strains are cultured in 250 ml of minimal medium (as above, without agar) with an addition of 15 mM of sodium pyruvate and 1 mM of 3-CB for 16 hours at 30° C.

The cells are harvested by centrifuging at 4° C, washed three times in 10 ml of cold (4° C) minimal medium (without carbon source), and recentrifuged, and finally suspended in such a quantity of minimal medium that an optical density of 30 is obtained at 420 nm. For the enzyme test of 2,4-D-monooxigenase and, respectively, 2,4-dichloro-phenolhydroxylase, 1 volume proportion of the cell suspension is mixed with 9 parts by volume of an oxygen-saturated minimal medium so that an optical density of 3 results at 420 nm. With the aid of a commercially available oxygen electrode, the oxygen absorption is then observed over 10 minutes without adding substrate. Subsequently, 2,4-D is added to a final concentration of 1 mM, or 2,4-dichlorophenol is added to a final concentration of 0.2 mM, and the decrease in oxygen concentration over a period of 20 minutes is observed. By means of this test, tfdA mutants can be distinguished from all other types of mutations, as well as from the wild-type strain JMP134: They show no increase whatever in oxygen consumption after addition of 2,4-D, whereas, upon addition of 2,4-dichlorophenol, a significant rise in oxygen absorption occurs. The wild-type strain, as well as tfdB mutants, show increased oxygen consumption after addition of 2,4-D as the substrate and thus indicate intact 2,4-D-monooxygenase. The two transposon mutants JMP134:Tn5-2 and JMP134:Tn5-4 are two 2,4-D-negative, 3-CB-positive mutants with a detectable 2,4-dichlorophenolhydroxylase, but without detectable 2,4-D-monooxygenase. They can thus be identified as tfdA mutants. This designation is confirmed by additional experiments described in the examples below.

Example 14: Expression of Cloned tfdA Genes in Gram-Negative Bacteria Other Than E. coli

(a) Preparation of Donor Strains for Conjugative Transfer of tfdA-Containing Plasmids

The plasmids constructed on the basis of mobilizable vectors with a wide hostrange, such as pVK101 [V.C. Knauf et al., Plasmid 8 :45 (1982)], pGSS33 [G.S. Sharpe, Gene 29 : 93 (1984)], and pKT321 [M. Bagdasarian et al., Current Topics in Microbiology and Immunology 96 : 74 (1982)], the preparation of which has been disclosed in Examples 1 through 7, can be transferred from the mobilizing strain E. coli S17-1 [Biotechnology 1 : 784 (1983)] by conjugation to other gram-negative bacteria, such as, for example, Alcaligenes eutrophus and Pseudomonas putida. For this purpose, they must first be introduced by transformation into E. coli S17-1 unless they have already been cloned in this strain. To this end, the plasmid DNA is isolated from the clones of E. coli LE392 containing the respective plasmid, in accordance with T. Maniatis et al. (see above). A mixture is made up of 0.2 ml of competent cells of E. coli LE392 produced according to T.J. Silhavy et al. (Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1984) and 1 µl of the isolated plasmid DNA, left on ice for 40 minutes, heated to 42° C for 3 minutes, mixed with 2 ml of LB medium, and incubated at 37° C for 60 minutes. Portions of 50 µl to 200 µl are spread on LB agar plates containing the corresponding antibiotic suitable for selection. The plates are incubated at 37° C for 16 hours.

(b) Conjugative Transfer

A thus-obtained strain of E. coli S17-7 containing one of the plasmids described in Examples 1-7 is cultured for 16 hours at 37° C in 5 ml of LB medium containing an antibiotic suitable for selection. As the recipient, one of the two tfdA mutants isolated in Example 13 (case A), the pJP4-free strain Alcaligenes eutrophus JMP222 [R.H. Don et al., J. Bacteriol. 145 : 681 (1981)] (case B), or Pseudomonas spec. B13 [E. Dorn et al., Arch. Microbiol. 99 : 61 (1974)] (case C) are cultured in 5 ml of PYE medium (3 g/l of peptone, 3 g/l of yeast extract) at 30° C for 16 hours. The cells of the donor strain are centrifuged and suspended in double the volume of PYE medium. The suspension of the donor cells and the culture of the recipient are mixed in equal parts. 100 µl of the mixture is pipetted onto a membrane filter (Millipore HA 0.45 µm) located on the surface of a PYE agar plate. Subsequently, the plate is incubated for 6 hours at 30° C. Then the cells are rinsed off the filter with 1 ml of 0.7% NaCl solution and the cell suspension is diluted in 7 steps respectively 1 : 10 (0.1 ml + 0.9 ml) with 0.7% NaCl solution. Of each dilution, 100 µl is spread on a minimal agar plate containing one of the following growth substrates: 1 mM 2,4-D in case A (tfdA mutant), 4 mM phenoxyacetic acid-Na in case B (Alcaligenes eutrophus JMP222) or 1 mM 4-chlorophenoxyacetic acid-Na in case C (Pseudomonas B13). The plates are incubated in case A for 14 days, in cases B and C for 4 days, at 30° C.

(c) Properties of the Newly Formed Strains

Mobilizable plasmids with a wide host range from the aforementioned group, containing an intact tfdA gene, render, after conjugative transfer, the respective recipient strain capable of synthesizing a functional 2,4-D-monooxygenase. This leads in case A (tfdA mutant) to complementation of the mutation and thus to restoration of the wild-type property with respect to utilization of 2,4-D, i.e. strains are produced which are 2,4-D-positive, growing on 2,4-D-minimal agar plates. The 2,4-D-monooxygenase coded by tfdA is furthermore capable of enzymatic conversion of, besides 2,4-D, also the compounds chemically related to 2,4-D, phenoxyacetic acid and 4-chlorophenoxyacetic acid, thus forming as the products phenol and, respectively, 4-chlorophenol. Since the strain Alcaligenes eutrophus JMP222 does exhibit genes for the complete metabolizing of phenol, there result after conjugative transfer of tfdA-containing plasmids, in case B, strains having the novel property of utilizing phenoxyacetic acid as growth substrate. In the same way, a tfdA-containing plasmid renders a Pseudomonas B13 equipped with this plasmid and having complete degradation pathways for phenol and 4-chlorophenol, capable of growing on the substrates phenoxyacetic acid and 4-chlorophenoxyacetic acid (case C). The test for utilization of a specific substrate as described above is especially suitable for the confirmation of expression of the tfdA gene by DNA fragments cloned in mobilizable broad host range vectors, and the shortened derivatives of these fragments, the production of which has been set forth in Examples 1-7. It turns out that the plasmids pVJH21, pGJS3, pKJS31, pKJS32, pKJSE330, pKJS32RHΔS' and pKJS(X)630 all contain an intact tfdA gene whereas the plasmid pKJEΔB130 does not code an operable gene product.

Example 15: Accumulation of tfdA-Coded 2,4-D-Monooxigenase in E. coli

(a) Production of Highly Productive E. coli Strains

The recombinant plasmids described in Examples 8, 9, 10 and 11, produced based on the vectors pT7-5 and pT7-6, constructed by Tabor, contain cloned DNA fragments following a strong promoter of phage T7. Plasmids from this group, containing an intact tfdA gene in the correct orientation with respect to the promoter, can produce 2,4-D-monooxygenase in large amounts under the influence of T7-RNA polymerase expressed by the plasmid pGP1-2 [S. Tabor et al., Proc. Natl. Acad. Sci. USA 82 : 1074 (1985)] in E. coli K38. Since the promoter is recognized specifically only by T7-RNA polymerase, and since the latter is on the plasmid pGP1-2 under the control of a heat-sensitive lamba repressor, the synthesis of the gene product can be induced by heat. By adding rifampicin, it is furthermore possible to inhibit the bacterial RNA polymerases. In order to produce the highly productive strains, the plasmid DNA is isolated from the plasmid-containing strains of E. coli LE392, obtained in Examples 8-11, according to T. Maniatis et al. (see above). E. coli K38, containing the plasmid pGP1-2, is cultured for 16 hours at 30° C in LB medium that has been combined with 50 µg/ml of kanamycin for selection oriented toward pGP1-2. From the grown culture, competent cells are produced according to the method by T.J. Silhavy et al. (Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1984). An amount of 0.2 ml of competent cells is mixed with 1 µl of the isolated DNA, left on ice for 40 minutes, heated to 37° C for 5 minutes, mixed with 2 ml of LB medium, and

incubated at 30° C for 60 minutes. Portions of 50 µl to 200 µl are spread on LB agar plates containing 50 µg/ml of ampicillin and 50 µg/ml of kanamycin. The plates are incubated at 30° C for 16 hours.

(b) Induced Production and Radiolabeling of the tfdA Gene Product

The thus-obtained ampicillin- and kanamycin-resistant clones contain the plasmid pGP1-2 and one of the recombinant pT7 derivatives. They are cultured in 10 ml of LB medium with an addition of 50 µg/ml of ampicillin and 50 µg/ml of kanamycin at 30° C. At an optical density of 0.5, measured at 590 nm, 0.2 ml of culture is removed by centrifuging. The cells are washed in 5 ml of M9 medium according to T. Maniatis et al. (see above), again centrifuged, and finally suspended in 1 ml of M9 medium containing 20 µg/ml of thiamine and respectively 0.01% of all proteinogenic amino acids except for methionine and cysteine (18 in total). The cell suspension is shaken at 30° C for 60 minutes; then the temperature is raised to 42° C. After 15 minutes, 10 µl of rifampicin solution (20 mg/ml in methanol) is added and the batch is left at 42° C for another 10 minutes. Thereafter the temperature is again lowered to 30° C; after 20 minutes the batch is combined with 10 µCi of L-(^{35}S)methionine (Amersham, cell labeling grade) and left for 5 minutes at room temperature. The cells are subsequently removed by centrifuging, suspended in 120 µl of application buffer (60 mM of Tris-HCl, pH 6.8, 1% sodium lauryl sulfate, 1% 2-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue), and heated to 95° C for 3 minutes.

(c) Identification of Specifically Radiolabeled tfdA Gene Product

The thus-obtained samples are loaded on a 12.5% strength polyacrylamide gel [U.K. Laemmli, Nature, 227 : 680 (1970)], and the entire bacterial proteins are separated electrophoretically in accordance with the method, known from the literature, described therein. After electrophoresis has been completed, the gel is stained for 30 minutes in a mixture of 2.5 g of SERVA blue G, 454 ml of methanol, 92 ml of acetic acid, and 454 ml of water and then decolorized for 24 hours in a mixture of 50 ml of methanol, 75 ml of acetic acid, and 875 ml of water. The gel is dried on a filter paper (Whatman 3MM) with the aid of a commercially available gel desiccant; then autoradiography is performed according to T. Maniatis et al. (see above) with an X-ray film (Kodak Industrex AX), and the film is developed. On the autoradiogram, an individual marked protein is identified in those strains containing, besides pGP1-2, one of the plasmids pTJSS'035, pTJS'B435, pTJS'X535 or pTJS'X535omega. All of these homologous plasmids have the feature in common that the cloned fragment is transcribed by the T7-RNA polymerase toward the SalI end. In case of plasmids having the reverse orientation of the insert (pTJSS'036, pTJS'B435 and pTJS'X535), no specifically marked protein is found. By comparison with standard proteins of a known size, the molecular weight of the marked proteins is determined. This results, for the gene products expressed by pTJSS'035, pTJS'B435 and pTJS'X535, in a molecular weight of 32,000, for the gene product expressed by pTJS'X535 in a molecular weight of 29,000.

(d) Induced Production with Confirmation of Enzymatic Activity

The above-obtained strains of E. coli K38, containing pGP1-2 and one of the plasmids pTJSS'035, pTJS'B435, pTJS'X535 or pTJS'X535omega, are grown at 30° C in 20 ml of LB medium with an addition of 50 µg/ml of ampicillin and 50 µg/ml of kanamycin. At an optical density of 1.0 at 590 nm, the temperature is increased to 42° C, and the culture is shaken for 25 minutes. Thereafter, 100 µl of rifampicin solution (20 mg/ml in methanol) is added and the culture shaken for 2 hours at 37° C. Detection of enzymatic activity of 2,4-D-monooxygenase in E. coli is carried out along the lines of the method of radioactive enzyme test described by Amy et al. [Appl. Env. Microbiol. 49 : 1237 (1985)], with the following modifications: The cells of an induced 20 ml culture are harvested by centrifuging, washed with 10 ml of EM medium, again centrifuged, and finally suspended in 10 ml of EM medium. The cell suspension is mixed in a 250 ml Warburg flask with 10 ml of transformation buffer, combined with 200 µg of unlabeled 2,4-D and 0.1 µCi of 2,4-dichlorophenoxy(2-^{14}C)acetic acid (Amersham, 55 mCi/mmol), and shaken in the sealed flask at 21° C for 4 hours. Subsequently, 0.5 ml of beta-phenylethylamine is pipetted into the central vessel of the Warburg flask, and 2 ml of 1-molar sulfuric acid is injected into the cell suspension. After one hour of shaking at 21° C, the beta-phenylethylamine is taken up in 5 ml of counter fluid ("rotiszint 22", Roth), and the radioactivity of the sample is measured in a scintillation counter. Strains of E. coli K38 containing, besides pGP1-2, one of the plasmids pTJSS'035, pTJS'B435 or pTJS'X535 exhibit high enzyme activity in this test, whereas those with the plasmid pTJS'X535omega express no enzyme activity.

Example 16: Determination of Base Sequence of DNA Cloned in M13

(a) Production of Deleted Phages from MJSS'030 and MJSS'031

For sequencing the 2-kilobase BamHI/SalI fragment of the recombinant phages according to the method by Sanger [Proc. Natl. Acad. Sci. USA, 74 : 5463 (1977)], a series of differently shortened phages is first produced by controlled deletion according to a process known from the literature [G. Henikoff, Gene, 28 : 351 (1984)], making it possible to determine the entire base sequence by overlapping sequencing of respectively 200 to 300 bases. For this purpose, the double-stranded DNA is isolated from strains of E. coli JM101 containing the phages MJSS'030 and, respectively, MJSS'031 produced in Example 12, in accordance with the method described therein for the phage vectors M13tg130 and M13tg131. 10 µg of double-stranded DNA of the phage MJSS'030 is cut with the enzymes PstI and SalI, 10 µg of double-stranded DNA of the phage MJSS'031 is cut with the enzymes BamHI and SacI. The restriction batches are extracted with phenol and the cut DNA is precipitated with ethanol. The subsequent digestion of DNA with exonuclease III and S1-nuclease, Klenow capping of the projecting DNA ends, and ligation take place exactly according to the method described by Henikoff, with the following change: Instead of using S1-nuclease, mung bean nuclease (Pharmacia) is utilized. Portions of 0.2 ml of competent cells of E. coli JM101 [J. Messing et al., Nucl. Acids Res. 9 : 309 (1981)], prepared according to the process by T.J. Silhavy et al. (Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1984), are combined with respectively 20 µl of the ligation batches, left on ice for 40 minutes, heated to 42° C for 3 minutes,

mixed with respectively 3 ml of melted (45° C) H-Top agar (Example 12), and poured on H-agar plates (Example 12). The plates are incubated at 37° C for 16 hours.

(b) Identification of Deleted Phages

E. coli JM101 is cultured in 2 ml of TY medium (Example 12) at 37° C for 16 hours. Of this culture, 1 ml is diluted with 100 ml of TY medium, and respectively 2 ml of the diluted cell suspension is infected with one of the above-obtained plaques. The cultures are shaken at 37° C for 5 hours and then centrifuged. From the supernatants of centrifugation, the single-strand DNA is obtained in accordance with the procedure indicated by Amersham [M13 Cloning and Sequencing Handbook, 1984]. The double-strand DNA is isolated from the centrifuged cells in accordance with T. Maniatis et al. (see above). The double-strand DNA of the shortened phages evolved from MJSS'030 is digested with BglII, that of the phages evolved from MJSS'031 with EcoRI and SalI. By subsequent electrophoretic separation of the fragments on a 2% agarose gel according to T. Maniatis et al. (see above), the size of the respective deletion is determined.

(c) Sequencing of Single-Strand DNA

The base sequence of the above-obtained single-strand DNA of the shortened phages, as well as of the single-strand DNA of MJSS'030 and MJSS'031 produced in Example 12 is determined according to the method, known from the literature, of dideoxynucleotide sequencing [Proc. Natl. Acad. Sci. USA 74 : 5463 (1977)]. In this procedure, the directions given by Amersham [M13 Cloning and Sequencing Handbook, 1984] are followed. In order to separate the DNA fragments, a gel having a length of 55 cm with a thickness increasing from 0.1 mm to 0.4 mm is utilized. The identified DNA sequences of

the shortened phages are joined with the aid of a computer program [C. Queen et al., Nucl. Acids Res. 12 : 581 (1984)] at overlapping regions to a DNA sequence encompassing the entire segment between the BamHI scission site and the SalI scission site of the insert cloned in M13tg130 and, respectively, M13tg131. The base sequence is confirmed by comparison of the two thus-obtained complementary DNA strands.

(d) Properties of Sequenced DNA

With the aid of the above-mentioned computer program, all properties of the DNA are determined which result directly from the base sequence. Among these, the most important that can be cited are: the position of the recognition sites for restriction endonucleases, the location and length of the open reading frames as possible coding regions of a gene, the frequency of specific bases and their distribution, and the occurrence of certain functional DNA sequences. Analysis of the DNA sequence shows an open reading frame, the location, length and transcription direction of which coincide with the properties of the tfdA gene, described in Examples 14 and 15. This frame begins at a GTG starting codon with the base No. 748 and ends upstream of a TAG stop codon with base No. 1608, and therefore has a length of 861 bases, corresponding to the length of the tfdA-coded protein (Example 15). The insertion of transcription and translation stop signals into the sole BglII cutting site of the sequenced fragment, as represented by the cloning of the omega fragment in pTJS'X535 (Example 11), shortens this open reading frame by calculation to 768 bases, which coincides with the expression of a shortened and enzymatically inactive gene product by the plasmid pTJS'X535omega (Example 15).

```
        10         20         30         40         50         60
GGATCCTGTCTCAGCTGGCGCGCAATGCTCGAACCCGCTGCGATATACAGCCGTTCGTAG

        70         80         90        100        110        120
TGCAGGTGCTCCACCGTGATTCCAGGCTCCTGGGGTAGAAGCGGCCGACACCGAGATGG

       130        140        150        160        170        180
ATGGTGCCGGCACGCAGGGCCTCGATCTGCCGCACCTTGGGCATCAGGGCCAGAGACAGC

       190        200        210        220        230        240
GTCGCCCCCGGGACCGCCTGCGTGAACGCATGGAGCAATGCCGGGACGGTCTGGTAGATC

       250        260        270        280        290        300
GCCGTGCCGAGGTAGCCGATATCGAGTTGGCCGATCTCGCCCCGGCTGGCGGCGCGGAC

       310        320        330        340        350        360
CGGTCCACGGAAGTCCGACCCAGTTCGAGCATGCGCCGTGCATCTTCGAGAAACGCGGCC

       370        380        390        400        410        420
CGGCGGGCGTGAGCTGCACGCCGCGCGCGCTGCGCTCGAACAACAACACGCCCAGATGC

       430        440        450        460        470        480
TGTTCGAGCGCGTGAATCTGTCGCGTGACCGGGGGCTGGGAAATATGCAGCCGCCCGCG

       490        500        510        520        530        540
GCGGCACCGACGTTGCCCTCCTCCGCGGCAGCAACGAAATAGCGAAGCTGTCGAAACTCC

       550        560        570        580        590        600
ATTCTTCACTCCTGGTGGCTGGCTCCGGCTGCCGGAGAGCCATACCGATCCCGTATCGCT

       610        620        630        640        650        660
CGCGCTGATGGAAGGTATTAGACCATATGGCCCGGCATTTCTAGACTACCGCCATGATAA

       670        680        690        700        710        720
AACTCGGCTGCTCTCTCGTCTGCTGGAACATCTTCAGGCGCGCTGAGCCGTCTTTTTGAA

       730        740        750        760        770        780
ACAGTCTCTTAGAAAAGGAGCAAAAAAGTGAGCGTCGTCGCAAATCCCCTTCATCCTCTT

       790        800        810        820        830        840
TTCGCCGCAGGGGTCGAAGACATCGACCTTCGAGAGGCCTTGGGTTCGACCGAGGTCCGA

       850        860        870        880        890        900
GAGATCGAACGGCTAATGGACGAGAAGTCGGTGCTGGTGTTCCGGGGGCAGCCCCTGAGT

       910        920        930        940        950        960
CAGGATCAGCAGATCGCCTTCGCGCGCAATTTCGGGCCACTCGAAGGCGGTTTCATCAAG

       970        980        990       1000       1010       1020
GTCAATCAAAGACCTTCGAGATTCAAGTACGCGGAGTTGGCGGACATCTCGAACGTCAGT

      1030       1040       1050       1060       1070       1080
CTCGACGGCAAGGTCGCGCAACGCGATGCGCGCGAGGTGGTCGGGAACTTCGCGAACCAG

      1090       1100       1110       1120       1130       1140
CTCTGGCACAGCGACAGCTCCTTTCAGCAACCTGCTGCCCGCTACTCGATGCTCTCCGCG

      1150       1160       1170       1180       1190       1200
GTGGTGGTTCCGCCGTCGGGCGGCGACACCGAGTTCTGCGACATGCGTGCGGCATACGAC

      1210       1220       1230       1240       1250       1260
GCGCTGCCTCGGGACCTCCAATCCGAGTTGGAAGGGCTGCGTGCCGAGCACTACGCACTG
```

FIG. 10a

```
        1270      1280      1290      1300      1310      1320
AACTCCCGCTTCCTGCTCGGCGACACCGACTATTCGGAAGCGCAACGCAATGCCATGCCG

        1330      1340      1350      1360      1370      1380
CCGGTCAACTGGCCGCTGGTTCGAACCCACGCCGGCTCCGGGCGCAAGTTTCTCTTCATC

        1390      1400      1410      1420      1430      1440
GGCGCGCACGCGAGCCACGTCGAAGGCCTTCCGGTGGCCGAAGGCCGGATGCTGCTTGCG

        1450      1460      1470      1480      1490      1500
GAGCTTCTCGAGCACGCGACACAGCGGGAATTCGTGTACCGGCATCGCTGGAACGTGGGA

        1510      1520      1530      1540      1550      1560
GATCTGGTGATGTGGGACAACCGCTGCGTTCTTCACCGCGGACGCAGGTACGACATCTCG

        1570      1580      1590      1600      1610      1620
GCCAGGCGTGAGCTGCGCCGGGCGACCACCCTGGACGATGCCGTCGTCTAGCGCACGCCA

        1630      1640      1650      1660      1670      1680
TGGCGCACGCCCTTTTCGCGAAGGCCCCACAAGATGTACGCAACCCTGATCAGCGGCAGC

        1690      1700      1710      1720      1730      1740
CGTAGCCTGGACGGCGACACCTTGGCGCAGCGCGTCCTTCGAGCGGCGGGCGGCCTGGCG

        1750      1760      1770      1780      1790      1800
GCATGGGGATTGAGGCCCGGTGATGTCGTCGCCATCCTCATGCGCAATGACTTTCCGGTG

        1810      1820      1830      1840      1850      1860
CTCGAAATGACGCTGGCCGCGAACCGCGCCGGCATCGTTGCGGTGCCTTTGAACTGGCAT

        1870      1880      1890      1900      1910      1920
GCGAACCGGGACGAGATCGCCTTCATCCTCGAGGACTGCAAAGCGCGTGTGCTCGTCGCG

        1930      1940      1950      1960      1970      1980
CACACCGATCTGCTCAAGGGCGTTGCATCCGCGGTGCCCGAGGCCTGCAAGGTGCTGGAA

        1990      2000      2010      2020      2030      2040
GCCGCGTCGCCGCCCGAGATCCGGCAGGCCTATCGGCTGTCCGATGCGTCGTGCACGGCG

        2050
AACCCGGGCACGGTCGAC
```

FIG. 10b

Form for the Deposit of Microorganisms

M3

International application:

MICROORGANISMS

Details of the microorganism referred to on page 13 line 12-26 of the description

A. IDENTIFICATION OF THE DEPOSIT[2]

Further deposits are identified on an additional sheet X [3]

Name of depository institution[4]

Deutsche Sammlung von Mikroorganismen (DSM)

Address of depository institution (including postal code and country)[4]

Gisebachstr. 8, D-3400 Göttingen

Date of deposit[5] 28.August 1986

Accession number[6] DSM 3829 - 3843

B. ADDITIONAL INDICATIONS[7] (The information is continued on a separate attached sheet ☐)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE[3] (if the indications are not for all

D. SEPARATE FURNISHING OF INDICATIONS[8]

The indications listed below will be submitted to the International Bureau later[9] (general designation of the indications e.g. "Accession number of Deposit")

E. ☐ This sheet was filed (to be checked by receiving office)

(signature) Dipl. Verwaltungswirt
(Authorised officer) R KONVALIN
Government Officer

☐ The date of the receipt (from the applicant) by the International Bureau[10]

(Authorised officer)

Form for the Deposit of Microorganisms M3

International application: PCT/DE 87/00932

MICROORGANISMS

Details of the microorganism referred to on page 13 line 12 of the description

A. IDENTIFICATION OF THE DEPOSIT[2]

Further deposits are identified on an additional sheet X [3]

Name of depository institution[4]

Deutsche Sammlung von Mikroorganismen (DSM)

Address of depository institution (including postal code and country)[4]

Gisebachstr. 8, D-3400 Göttingen, Germany

Date of deposit[5] 28.08.1986

Accession number[6] DSM 3829

B. ADDITIONAL INDICATIONS[7] (The information is continued on a separate attached sheet ☐)

For those deposits for which a European Patent is applied for, up to the publication of the of the mention of grant of the European Patent or until the date on which the application has been refused or withdrawn or deemed to be withdrawn, the availability to the deposited microorganism shall be effected only through delivery of a sample of the microorganism to an expertwho is named by the person who has requested the sample. (Rule 28, paragraph 4, EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE[3] (if the indications are not for all

D. SEPARATE FURNISHING OF INDICATIONS[8]

The indications listed below will be submitted to the International Bureau later[9] (general designation of the indications e.g. "Accession number of Deposit")

E. ☐ This sheet was filed (to be checked by receiving office)

(signature) Dipl. Verwaltungswirt
(Authorised officer)
R KONVALIN
Government Officer

☐ The date of the receipt (from the applicant) by the International Bureau[10]

(Authorised officer)

Form for the Deposit of Microorganisms M3

International application: PCT/DE 87/00932

MICROORGANISMS

Details of the microorganism referred to on page 13 line 13 of the description

A. IDENTIFICATION OF THE DEPOSIT[2]

Further deposits are identified on an additional sheet X [3]

Name of depository institution[4]

Deutsche Sammlung von Mikroorganismen (DSM)

Address of depository institution (including postal code and country)[4]

Gisebachstr. 8, D-3400 Göttingen, Germany

Date of deposit[5] 28.08.1986

Accession number[6] DSM 3830

B. ADDITIONAL INDICATIONS[7] (The information is continued on a separate attached sheet ☐)

For those deposits for which a European Patent is applied for, up to the publication of the of the mention of grant of the European Patent or until the date on which the application has been refused or withdrawn or deemed to be withdrawn, the availability to the deposited microorganism shall be effected only through delivery of a sample of the microorganism to an expertwho is named by the person who has requested the sample. (Rule 28, paragraph 4, EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE[3] (if the indications are not for all

D. SEPARATE FURNISHING OF INDICATIONS[8]

The indications listed below will be submitted to the International Bureau later[9] (general designation of the indications e.g. "Accession number of Deposit")

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(Authorised officer) R KONVALIN
Government Officer

☐ The date of the receipt (from the applicant) by the International Bureau[10]

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Form for the Deposit of Microorganisms

M3

International application: PCT/DE 87/00932

MICROORGANISMS

Details of the microorganism referred to on page 13 line 14 of the description

A. IDENTIFICATION OF THE DEPOSIT[2]

Further deposits are identified on an additional sheet X [3]

Name of depository institution[4]

Deutsche Sammlung von Mikroorganismen (DSM)

Address of depository institution (including postal code and country)[4]

Gisebachstr. 8, D-3400 Göttingen, Germany

Date of deposit[5]	Accession number[6]
28.08.1986	DSM 3831

B. ADDITIONAL INDICATIONS[7] (The information is continued on a separate attached sheet ☐)

For those deposits for which a European Patent is applied for, up to the publication of the of the mention of grant of the European Patent or until the date on which the application has been refused or withdrawn or deemed to be withdrawn, the availability to the deposited microorganism shall be effected only through delivery of a sample of the microorganism to an expertwho is named by the person who has requested the sample. (Rule 28, paragraph 4, EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE[3] (if the indications are not for all

D. SEPARATE FURNISHING OF INDICATIONS[8]

The indications listed below will be submitted to the International Bureau later[9] (general designation of the indications e.g. "Accession number of Deposit")

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(signature) Dipl. Verwaltungswirt

(Authorised officer) R KONVALIN

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Form for the Deposit of Microorganisms M3

International application: PCT/DE 87/00932

MICROORGANISMS

Details of the microorganism referred to on page 13 line 15 of the description

A. IDENTIFICATION OF THE DEPOSIT[2]

Further deposits are identified on an additional sheet X [3]

Name of depository institution[4]

Deutsche Sammlung von Mikroorganismen (DSM)

Address of depository institution (including postal code and country)[4]

Gisebachstr. 8, D-3400 Göttingen, Germany

Date of deposit[5]	Accession number[6]
28.08.1986	DSM 3832

B. ADDITIONAL INDICATIONS[7] (The information is continued on a separate attached sheet ☐)

For those deposits for which a European Patent is applied for, up to the publication of the of the mention of grant of the European Patent or until the date on which the application has been refused or withdrawn or deemed to be withdrawn, the availability to the deposited microorganism shall be effected only through delivery of a sample of the microorganism to an expertwho is named by the person who has requested the sample. (Rule 28, paragraph 4, EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE[3] (if the indications are not for all

D. SEPARATE FURNISHING OF INDICATIONS[8]

The indications listed below will be submitted to the International Bureau later[9] (general designation of the indications e.g. "Accession number of Deposit")

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(Authorised officer) R KONVALIN
Government Officer

☐ The date of the receipt (from the applicant) by the International Bureau[10]

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Form for the Deposit of Microorganisms

M3

International application: PCT/DE 87/00932

MICROORGANISMS

Details of the microorganism referred to on page 13 line 16 of the description

A. IDENTIFICATION OF THE DEPOSIT[2]

Further deposits are identified on an additional sheet X [3]

Name of depository institution[4]

Deutsche Sammlung von Mikroorganismen (DSM)

Address of depository institution (including postal code and country)[4]

Gisebachstr. 8, D-3400 Göttingen, Germany

Date of deposit[5]	Accession number[6]
28.08.1986	DSM 3833

B. ADDITIONAL INDICATIONS[7] (The information is continued on a separate attached sheet ☐)

For those deposits for which a European Patent is applied for, up to the publication of the of the mention of grant of the European Patent or until the date on which the application has been refused or withdrawn or deemed to be withdrawn, the availability to the deposited microorganism shall be effected only through delivery of a sample of the microorganism to an expertwho is named by the person who has requested the sample. (Rule 28, paragraph 4, EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE[3] (if the indications are not for all

D. SEPARATE FURNISHING OF INDICATIONS[8]

The indications listed below will be submitted to the International Bureau later[9] (general designation of the indications e.g. "Accession number of Deposit")

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(Authorised officer)

R KONVALIN

Government Officer

☐ The date of the receipt (from the applicant) by the International Bureau[10]

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Form for the Deposit of Microorganisms

M3

International application: PCT/DE 87/00932

MICROORGANISMS

Details of the microorganism referred to on page 13 line 17 of the description

A. IDENTIFICATION OF THE DEPOSIT[2]

Further deposits are identified on an additional sheet X [3]

Name of depository institution[4]

Deutsche Sammlung von Mikroorganismen (DSM)

Address of depository institution (including postal code and country)[4]

Gisebachstr. 8, D-3400 Göttingen, Germany

Date of deposit[5]	Accession number[6]
28.08.1986	DSM 3834

B. ADDITIONAL INDICATIONS[7] (The information is continued on a separate attached sheet ☐)

For those deposits for which a European Patent is applied for, up to the publication of the of the mention of grant of the European Patent or until the date on which the application has been refused or withdrawn or deemed to be withdrawn, the availability to the deposited microorganism shall be effected only through delivery of a sample of the microorganism to an expertwho is named by the person who has requested the sample. (Rule 28, paragraph 4, EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE[3] (if the indications are not for all

D. SEPARATE FURNISHING OF INDICATIONS[8]

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(signature) Dipl. Verwaltungswirt
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R KONVALIN
Government Officer

☐ The date of the receipt (from the applicant) by the International Bureau[10]

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Form for the Deposit of Microorganisms

M3

International application: PCT/DE 87/00932

MICROORGANISMS

Details of the microorganism referred to on page 13 line 18 of the description

A. IDENTIFICATION OF THE DEPOSIT[2]

Further deposits are identified on an additional sheet X [3]

Name of depository institution[4]

Deutsche Sammlung von Mikroorganismen (DSM)

Address of depository institution (including postal code and country)[4]

Gisebachstr. 8, D-3400 Göttingen, Germany

Date of deposit[5] 28.08.1986

Accession number[6] DSM 3835

B. ADDITIONAL INDICATIONS[7] (The information is continued on a separate attached sheet ☐)

For those deposits for which a European Patent is applied for, up to the publication of the of the mention of grant of the European Patent or until the date on which the application has been refused or withdrawn or deemed to be withdrawn, the availability to the deposited microorganism shall be effected only through delivery of a sample of the microorganism to an expertwho is named by the person who has requested the sample. (Rule 28, paragraph 4, EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE[3] (if the indications are not for all

D. SEPARATE FURNISHING OF INDICATIONS[8]

The indications listed below will be submitted to the International Bureau later[9] (general designation of the indications e.g. "Accession number of Deposit")

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R KONVALIN
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Form for the Deposit of Microorganisms

M3

International application: PCT/DE 87/00932

MICROORGANISMS

Details of the microorganism referred to on page 13 line 19 of the description

A. IDENTIFICATION OF THE DEPOSIT[2]

Further deposits are identified on an additional sheet X [3]

Name of depository institution[4]

Deutsche Sammlung von Mikroorganismen (DSM)

Address of depository institution (including postal code and country)[4]

Gisebachstr. 8, D-3400 Göttingen, Germany

Date of deposit[5] 28.08.1986

Accession number[6] DSM 3836

B. ADDITIONAL INDICATIONS[7] (The information is continued on a separate attached sheet ☐)

For those deposits for which a European Patent is applied for, up to the publication of the of the mention of grant of the European Patent or until the date on which the application has been refused or withdrawn or deemed to be withdrawn, the availability to the deposited microorganism shall be effected only through delivery of a sample of the microorganism to an expertwho is named by the person who has requested the sample. (Rule 28, paragraph 4, EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE[3] (if the indications are not for all

D. SEPARATE FURNISHING OF INDICATIONS[8]

The indications listed below will be submitted to the International Bureau later[9] (general designation of the indications e.g. "Accession number of Deposit")

E. ☐ This sheet was filed (to be checked by receiving office)

(signature) Dipl. Verwaltungswirt

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R KONVALIN
Government Officer

☐ The date of the receipt (from the applicant) by the International Bureau[10]

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Form for the Deposit of Microorganisms

M3

International application: PCT/DE 87/00932

MICROORGANISMS

Details of the microorganism referred to on page 13 line 20 of the description

A. IDENTIFICATION OF THE DEPOSIT[2]

Further deposits are identified on an additional sheet X [3]

Name of depository institution[4]

Deutsche Sammlung von Mikroorganismen (DSM)

Address of depository institution (including postal code and country)[4]

Gisebachstr. 8, D-3400 Göttingen, Germany

Date of deposit[5]	Accession number[6]
28.08.1986	DSM 3837

B. ADDITIONAL INDICATIONS[7] (The information is continued on a separate attached sheet ☐)

For those deposits for which a European Patent is applied for, up to the publication of the of the mention of grant of the European Patent or until the date on which the application has been refused or withdrawn or deemed to be withdrawn, the availability to the deposited microorganism shall be effected only through delivery of a sample of the microorganism to an expertwho is named by the person who has requested the sample. (Rule 28, paragraph 4, EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE[3] (if the indications are not for all

D. SEPARATE FURNISHING OF INDICATIONS[8]

The indications listed below will be submitted to the International Bureau later[9] (general designation of the indications e.g. "Accession number of Deposit")

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(signature) Dipl. Verwaltungswirt
(Authorised officer) R KONVALIN
Government Officer

☐ The date of the receipt (from the applicant) by the International Bureau[10]

(Authorised officer)

Form for the Deposit of Microorganisms

M3

International application: PCT/DE 87/00932

MICROORGANISMS

Details of the microorganism referred to on page 13 line 21 of the description

A. IDENTIFICATION OF THE DEPOSIT[2]

Further deposits are identified on an additional sheet X [3]

Name of depository institution[4]

Deutsche Sammlung von Mikroorganismen (DSM)

Address of depository institution (including postal code and country)[4]

Gisebachstr. 8, D-3400 Göttingen, Germany

Date of deposit[5]	Accession number[6]
28.08.1986	DSM 3838

B. ADDITIONAL INDICATIONS[7] (The information is continued on a separate attached sheet ☐)

For those deposits for which a European Patent is applied for, up to the publication of the of the mention of grant of the European Patent or until the date on which the application has been refused or withdrawn or deemed to be withdrawn, the availability to the deposited microorganism shall be effected only through delivery of a sample of the microorganism to an expertwho is named by the person who has requested the sample. (Rule 28, paragraph 4, EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE[3] (if the indications are not for all

D. SEPARATE FURNISHING OF INDICATIONS[8]

The indications listed below will be submitted to the International Bureau later[9] (general designation of the indications e.g. "Accession number of Deposit")

E. ☐ This sheet was filed (to be checked by receiving office)

(signature) Dipl. Verwaltungswirt

(Authorised officer) R KONVALIN

Government Officer

☐ The date of the receipt (from the applicant) by the International Bureau[10]

(Authorised officer)

Form for the Deposit of Microorganisms M3

International application: PCT/DE 87/00932

MICROORGANISMS

Details of the microorganism referred to on page 13 line 22 of the description

A. IDENTIFICATION OF THE DEPOSIT[2]

Further deposits are identified on an additional sheet X [3]

Name of depository institution[4]

Deutsche Sammlung von Mikroorganismen (DSM)

Address of depository institution (including postal code and country)[4]

Gisebachstr. 8, D-3400 Göttingen, Germany

Date of deposit[5] 28.08.1986

Accession number[6] DSM 3839

B. ADDITIONAL INDICATIONS[7] (The information is continued on a separate attached sheet ☐)

For those deposits for which a European Patent is applied for, up to the publication of the of the mention of grant of the European Patent or until the date on which the application has been refused or withdrawn or deemed to be withdrawn, the availability to the deposited microorganism shall be effected only through delivery of a sample of the microorganism to an expertwho is named by the person who has requested the sample. (Rule 28, paragraph 4, EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE[3] (if the indications are not for all

D. SEPARATE FURNISHING OF INDICATIONS[8]

The indications listed below will be submitted to the International Bureau later[9] (general designation of the indications e.g. "Accession number of Deposit")

E. ☐ This sheet was filed (to be checked by receiving office)

(signature) Dipl. Verwaltungswirt
(Authorised officer) R KONVALIN
Government Officer

☐ The date of the receipt (from the applicant) by the International Bureau[10]

(Authorised officer)

Form for the Deposit of Microorganisms

M3

International application: PCT/DE 87/00932

MICROORGANISMS

Details of the microorganism referred to on page 13 line 23 of the description

A. IDENTIFICATION OF THE DEPOSIT[2]

Further deposits are identified on an additional sheet X [3]

Name of depository institution[4]

Deutsche Sammlung von Mikroorganismen (DSM)

Address of depository institution (including postal code and country)[4]

Gisebachstr. 8, D-3400 Göttingen, Germany

Date of deposit[5]	Accession number[6]
28.08.1986	DSM 3840

B. ADDITIONAL INDICATIONS[7] (The information is continued on a separate attached sheet ☐)

For those deposits for which a European Patent is applied for, up to the publication of the of the mention of grant of the European Patent or until the date on which the application has been refused or withdrawn or deemed to be withdrawn, the availability to the deposited microorganism shall be effected only through delivery of a sample of the microorganism to an expertwho is named by the person who has requested the sample. (Rule 28, paragraph 4, EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE[3] (if the indications are not for all

D. SEPARATE FURNISHING OF INDICATIONS[8]

The indications listed below will be submitted to the International Bureau later[9] (general designation of the indications e.g. "Accession number of Deposit")

E. ☐ This sheet was filed (to be checked by receiving office)

(signature) Dipl. Verwaltungswirt
(Authorised officer) R KONVALIN
Government Officer

☐ The date of the receipt (from the applicant) by the International Bureau[10]

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Form for the Deposit of Microorganisms M3

International application: PCT/DE 87/00932

MICROORGANISMS

Details of the microorganism referred to on page 13 line 24 of the description

A. IDENTIFICATION OF THE DEPOSIT[2]

Further deposits are identified on an additional sheet X [3]

Name of depository institution[4]

Deutsche Sammlung von Mikroorganismen (DSM)

Address of depository institution (including postal code and country)[4]

Gisebachstr. 8, D-3400 Göttingen, Germany

Date of deposit[5] 28.08.1986

Accession number[6] DSM 3841

B. ADDITIONAL INDICATIONS[7] (The information is continued on a separate attached sheet ☐)

For those deposits for which a European Patent is applied for, up to the publication of the of the mention of grant of the European Patent or until the date on which the application has been refused or withdrawn or deemed to be withdrawn, the availability to the deposited microorganism shall be effected only through delivery of a sample of the microorganism to an expertwho is named by the person who has requested the sample. (Rule 28, paragraph 4, EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE[3] (if the indications are not for all

D. SEPARATE FURNISHING OF INDICATIONS[8]

The indications listed below will be submitted to the International Bureau later[9] (general designation of the indications e.g. "Accession number of Deposit")

E. ☐ This sheet was filed (to be checked by receiving office)

(signature) Dipl. Verwaltungswirt

(Authorised officer) R KONVALIN

Government Officer

☐ The date of the receipt (from the applicant) by the International Bureau[10]

(Authorised officer)

Form for the Deposit of Microorganisms M3

International application: PCT/DE 87/00932

MICROORGANISMS

Details of the microorganism referred to on page 13 line 25 of the description

A. IDENTIFICATION OF THE DEPOSIT[2]

Further deposits are identified on an additional sheet X [3]

Name of depository institution[4]

Deutsche Sammlung von Mikroorganismen (DSM)

Address of depository institution (including postal code and country)[4]

Gisebachstr. 8, D-3400 Göttingen, Germany

Date of deposit[5]	Accession number[6]
28.08.1986	DSM 3842

B. ADDITIONAL INDICATIONS[7] (The information is continued on a separate attached sheet ☐)

For those deposits for which a European Patent is applied for, up to the publication of the of the mention of grant of the European Patent or until the date on which the application has been refused or withdrawn or deemed to be withdrawn, the availability to the deposited microorganism shall be effected only through delivery of a sample of the microorganism to an expertwho is named by the person who has requested the sample. (Rule 28, paragraph 4, EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE[3] (if the indications are not for all

D. SEPARATE FURNISHING OF INDICATIONS[8]

The indications listed below will be submitted to the International Bureau later[9] (general designation of the indications e.g. "Accession number of Deposit")

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Form for the Deposit of Microorganisms

M3

International application: PCT/DE 87/00932

MICROORGANISMS

Details of the microorganism referred to on page 13 line 26 of the description

A. IDENTIFICATION OF THE DEPOSIT[2]

Further deposits are identified on an additional sheet X [3]

Name of depository institution[4]

Deutsche Sammlung von Mikroorganismen (DSM)

Address of depository institution (including postal code and country)[4]

Gisebachstr. 8, D-3400 Göttingen, Germany

Date of deposit[5]	Accession number[6]
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B. ADDITIONAL INDICATIONS[7] (The information is continued on a separate attached sheet ☐)

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(signature) Dipl. Verwaltungswirt
(Authorised officer)
R KONVALIN
Government Officer

☐ The date of the receipt (from the applicant) by the International Bureau[10]

(Authorised officer)

Patent claims

1. tfdA-2,4-monooxygenase-gene mutant Alkaligenes eutrophus JMP 134:Tn 5-2 (DSM 3842).

2. tfdA-2,4-monooxygenase-gene mutant Alkaligenes eutrophus JMP 134:Tn 5-4 (DSM 3843).

3. Use of tfdA-2,4-monooxygenase-gene mutant Alkaligenes eutrophus JMP 134:Tn 5-2 and JMP 134:Tn 5-4 for the identification and isolation of the plasmid containing the tfdA-genes.

4. Recombinant DNA, characterised in that it comprises the base sequence 1 to 861, coding for the 2,4-D degrading protein with the specified amino acid sequence 1 to 287,

```
        .         .        30         .         .        60
GTGAGCGTCGTCGCAAATCCCCTTCATCCTCTTTTCGCCGCAGGGGTCGAAGACATCGAC
ValSerValValAlaAsnProLeuHisProLeuPheAlaAlaGlyValGluAspIleAsp

        .         .        90         .         .       120
CTTCGAGAGGCCTTGGGTTCGACCGAGGTCCGAGAGATCGAACGGCTAATGGACGAGAAG
LeuArgGluAlaLeuGlySerThrGluValArgGluIleGluArgLeuMetAspGluLys

        .         .       150         .         .       180
TCGGTGCTGGTGTTCCGGGGGCAGCCCCTGAGTCAGGATCAGCAGATCGCCTTCGCGCGC
SerValLeuValPheArgGlyGlnProLeuSerGlnAspGlnGlnIleAlaPheAlaArg

        .         .       210         .         .       240
AATTTCGGGCCACTCGAAGGCGGTTTCATCAAGGTCAATCAAAGACCTTCGAGATTCAAG
AsnPheGlyProLeuGluGlyGlyPheIleLysValAsnGlnArgProSerArgPheLys

        .         .       270         .         .       300
TACGCGGAGTTGGCGGACATCTCGAACGTCAGTCTCGACGGCAAGGTCGCGCAACGCGAT
TyrAlaGluLeuAlaAspIleSerAsnValSerLeuAspGlyLysValAlaGlnArgAsp

        .         .       330         .         .       360
GCGCGCGAGGTGGTCGGGAACTTCGCGAACCAGCTCTGGCACAGCGACAGCTCCTTTCAG
AlaArgGluValValGlyAsnPheAlaAsnGlnLeuTrpHisSerAspSerSerPheGln

        .         .       390         .         .       420
CAACCTGCTGCCCGCTACTCGATGCTCTCCGCGGTGGTGGTTCCGCCGTCGGGCGGCGAC
GlnProAlaAlaArgTyrSerMetLeuSerAlaValValValProProSerGlyGlyAsp

        .         .       450         .         .       480
ACCGAGTTCTGCGACATGCGTGCGGCATACGACGCGCTGCCTCGGGACCTCCAATCCGAG
ThrGluPheCysAspMetArgAlaAlaTyrAspAlaLeuProArgAspLeuGlnSerGlu
```

```
                          510                 540
TTGGAAGGGCTGCGTGCCGAGCACTACGCACTGAACTCCCGCTTCCTGCTCGGCGACACC
LeuGluGlyLeuArgAlaGluHisTyrAlaLeuAsnSerArgPheLeuLeuGlyAspThr

                          570                 600
GACTATTCGGAAGCGCAACGCAATGCCATGCCGCCGGTCAACTGGCCGCTGGTTCGAACC
AspTyrSerGluAlaGlnArgAsnAlaMetProProValAsnTrpProLeuValArgThr

                          630                 660
CACGCCGGCTCCGGGCGCAAGTTTCTCTTCATCGGCGCGCACGCGAGCCACGTCGAAGGC
HisAlaGlySerGlyArgLysPheLeuPheIleGlyAlaHisAlaSerHisValGluGly

                          690                 720
CTTCCGGTGGCCGAAGGCCGGATGCTGCTTGCGGAGCTTCTCGAGCACGCGACACAGCGG
LeuProValAlaGluGlyArgMetLeuLeuAlaGluLeuLeuGluHisAlaThrGlnArg

                          750                 780
GAATTCGTGTACCGGCATCGCTGGAACGTGGGAGATCTGGTGATGTGGGACAACCGCTGC
GluPheValTyrArgHisArgTrpAsnValGlyAspLeuValMetTrpAspAsnArgCys

                          810                 840
GTTCTTCACCGCGGACGCAGGTACGACATCTCGGCCAGGCGTGAGCTGCGCCGGGCGACC
ValLeuHisArgGlyArgArgTyrAspIleSerAlaArgArgGluLeuArgArgAlaThr

ACCCTGGACGATGCCGTCGTC
ThrLeuAspAspAlaValVal
```

or an equivalent thereof which codes, according to the genetic code, for the same amino acid sequence.

5. Plasmids containing a gene or subfragments of a gene according to claim 4.

6. Plasmid pVJH21, prepared according to Example 1 from the plasmids pJP4 and pVK101.

7. Plasmid pGJS3, prepared according to Example 2 from the plasmids PVJH21 and PGSS33.

8. Plasmid pKJS31, prepared from the plasmids pGJS3 and pKT231 (deposited in E. coli S17-1, DSM 3835).

9. Plasmid pKJ32, prepared according to Example 3 from the plasmids pGJS3 and pKT231.

10. Plasmid pKJSB330, prepared according to Example 4, from the plasmid pKJS31.

11. Plasmid pKJS(X)630, prepared according to Example 10 (deposited in E. coli S17-1, DSM 3837).

12. Plasmid pKJEΔB130, prepared according to Example 6 from the plasmids pKT231 and pKJS32.

13. Plasmid pTJS'B435, prepared according to Example 9 from the plasmids pT7-5 and pKJSB330.

14. Plasmid pTJS'B436, prepared according to Example 9 from the plasmids pT7-6 and pKJSB330.

15. Plasmid pKJS32RH S', prepared from the plasmids pKJS32 and pRME1 (deposited in E. coli S17-1, DSM 3836).

16. Plasmid pTJSS'035, prepared from the plasmids pT7-5 and pKJS32 (deposited in E. coli LE 392, DSM 3832).

17. Plasmid pTJSS'036, prepared according to Example 8 from the plasmids pT7-6 and pKJS32.

18. Plasmid pTJS'535, prepared from the plasmids pT7-5 and pTJSS'035 (deposited in E. coli K38 (pGT1-2/pTJSX535), DSM 3839).

19. Plasmid pTJS'X536, prepared according to Example 10 from the plasmids pT7-6 and pTJSS'035.

20. Plasmid pTJS'X535omega, prepared according to Example

11 from the plasmids pTJS'X535 and pDOC37.

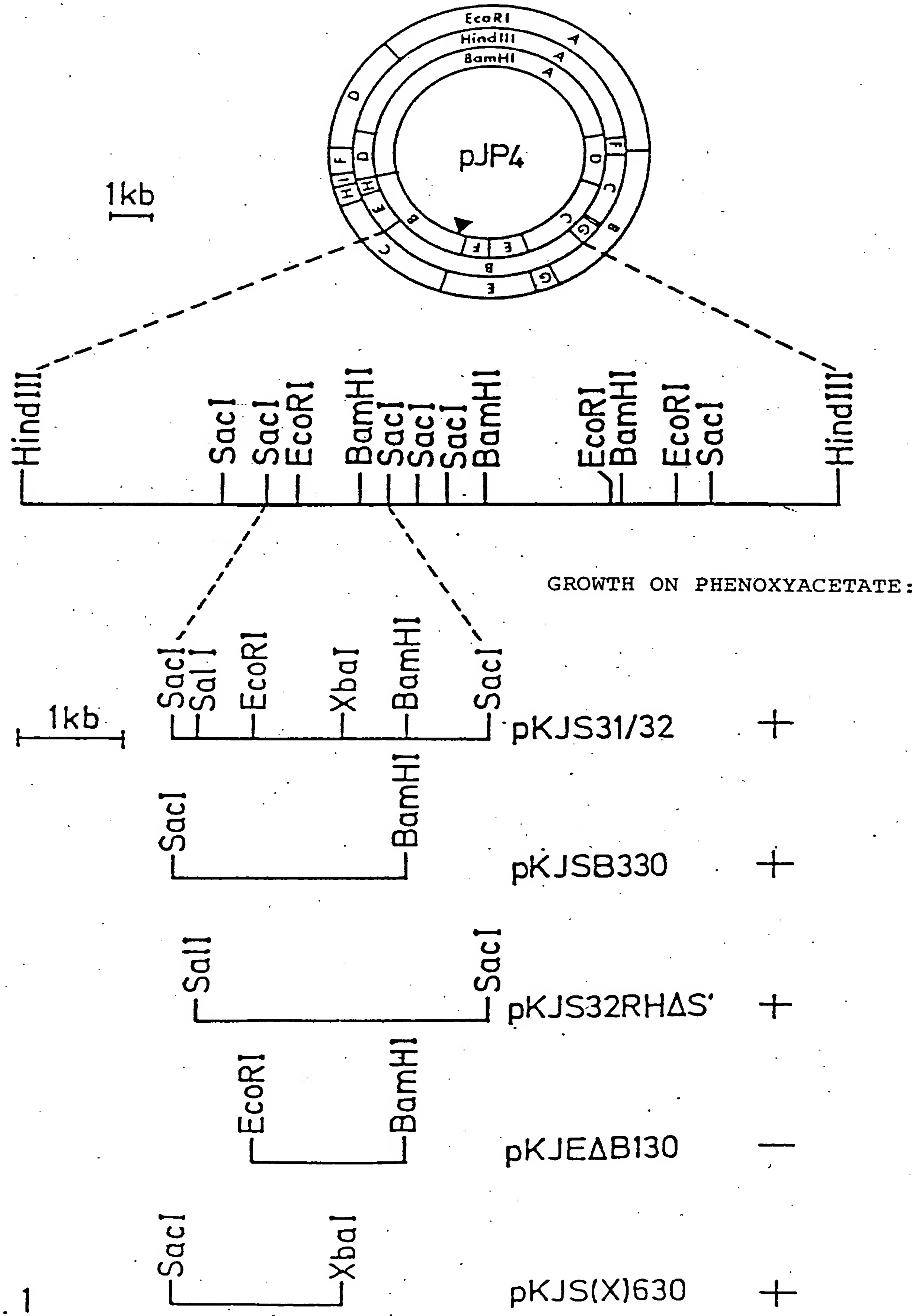
21. Phages MJSS'030, prepared according to Example 12 from the phage M13tg130 and the plasmid pKJS32.

22. Phages MJSS'031, prepared according to Example 12 from the phage M13tg131 and the plasmid pKJS32.

23. E. coli strains containing plasmids according to claim 5.

24. Pseudomonas strains containing plasmids according to claim 5, characterised in that these strains can utilise 4-chlorophenoxyacetic acid

25. Use of tfDA-genes or fragments of these genes comprising plasmids as the starting material for the preparation of 2,4-D-degrading plants, characterised in that these plants are resistant to the growth inhibition activity of 2,4-D.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BamHI
SacI
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BamHI
EcoRI
BamHI
EcoRI
SacI
HindIII
GROWTH ON PHENOXYACETATE:
SacI
SalI
EcoRI
XbaI
BamHI
SacI
1kb
pKJS31/32 +
SacI
BamHI
pKJSB330 +
SalI
SacI
pKJS32RHΔS' +
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BamHI
pKJEΔB130 —
SacI
XbaI
pKJS(X)630 +

FIG. 1

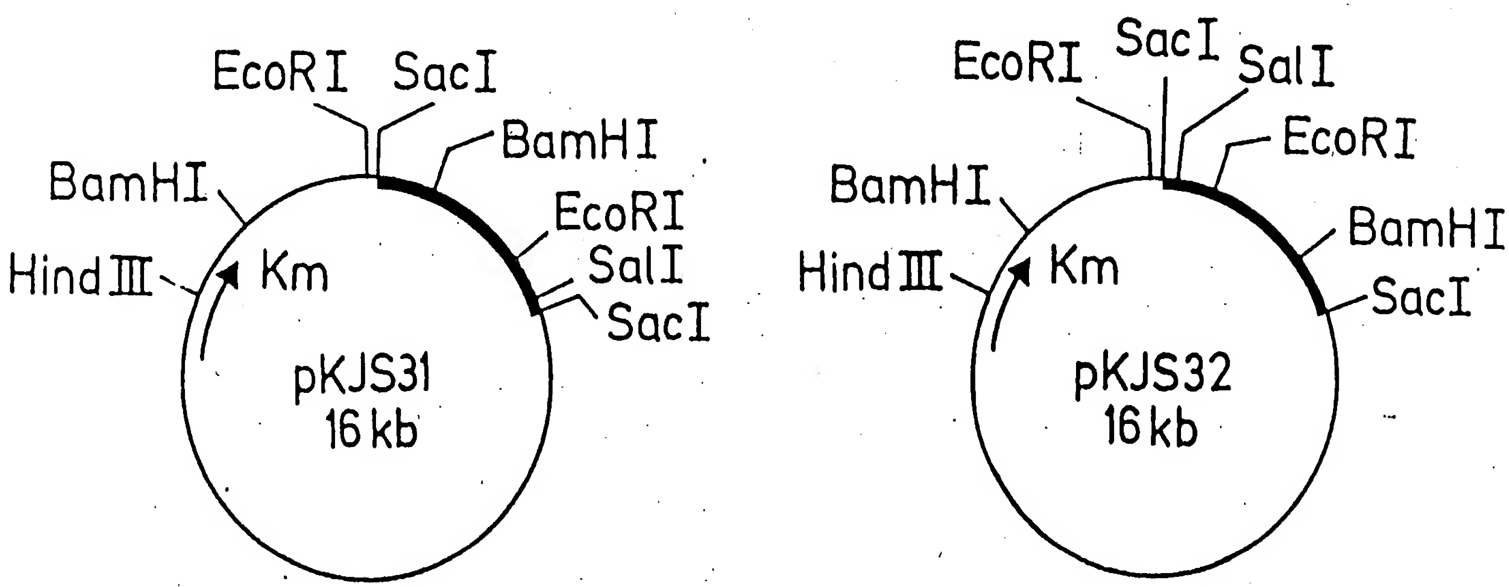
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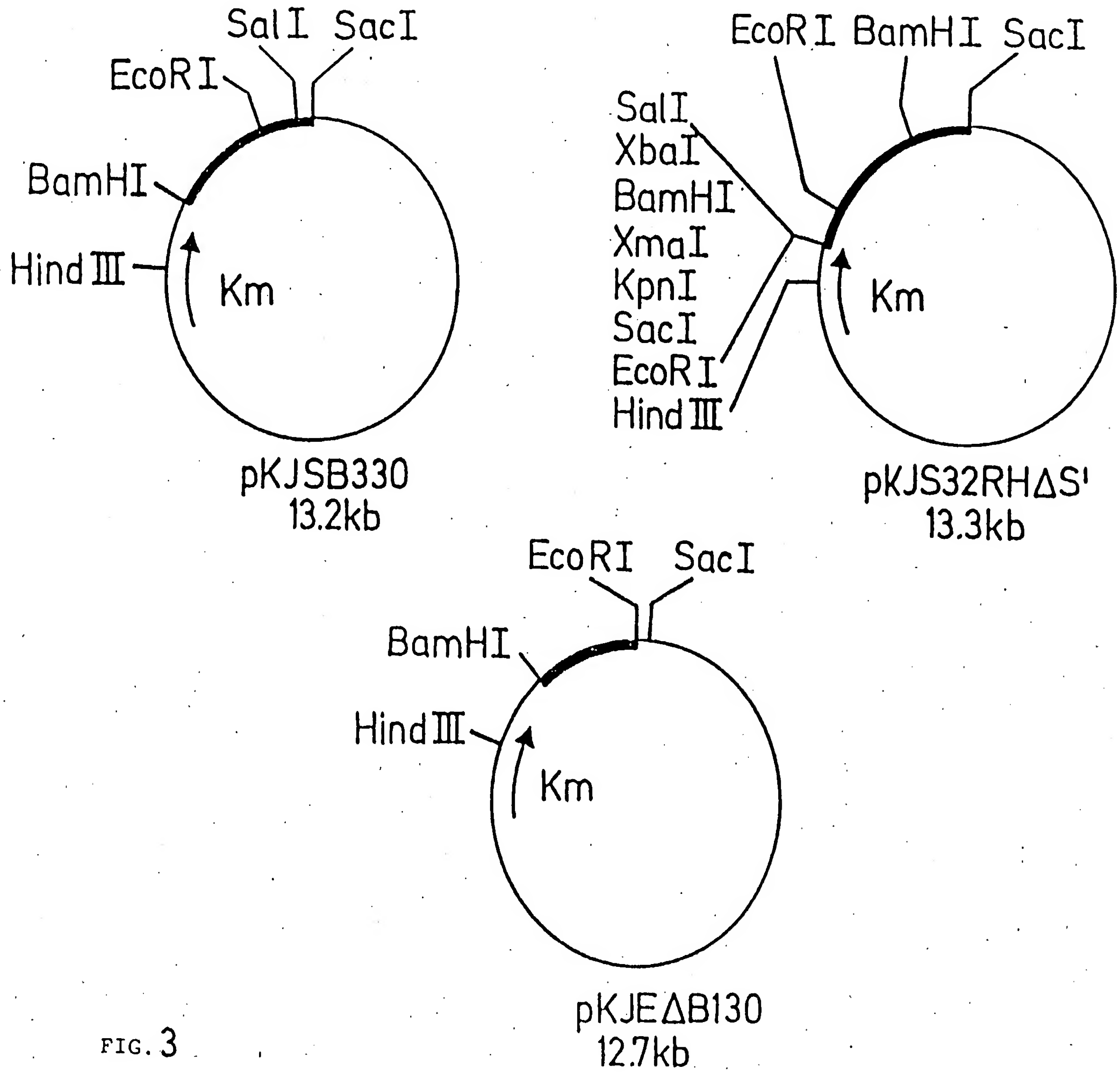
FIG. 2



Sal I
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Sal I
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Xma I
Kpn I
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FIG. 3

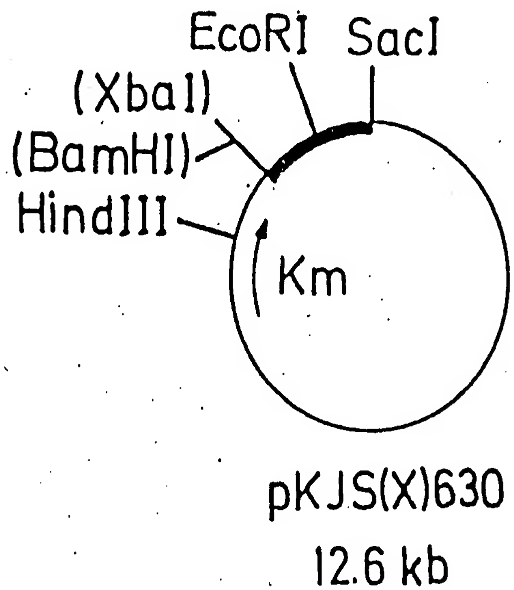
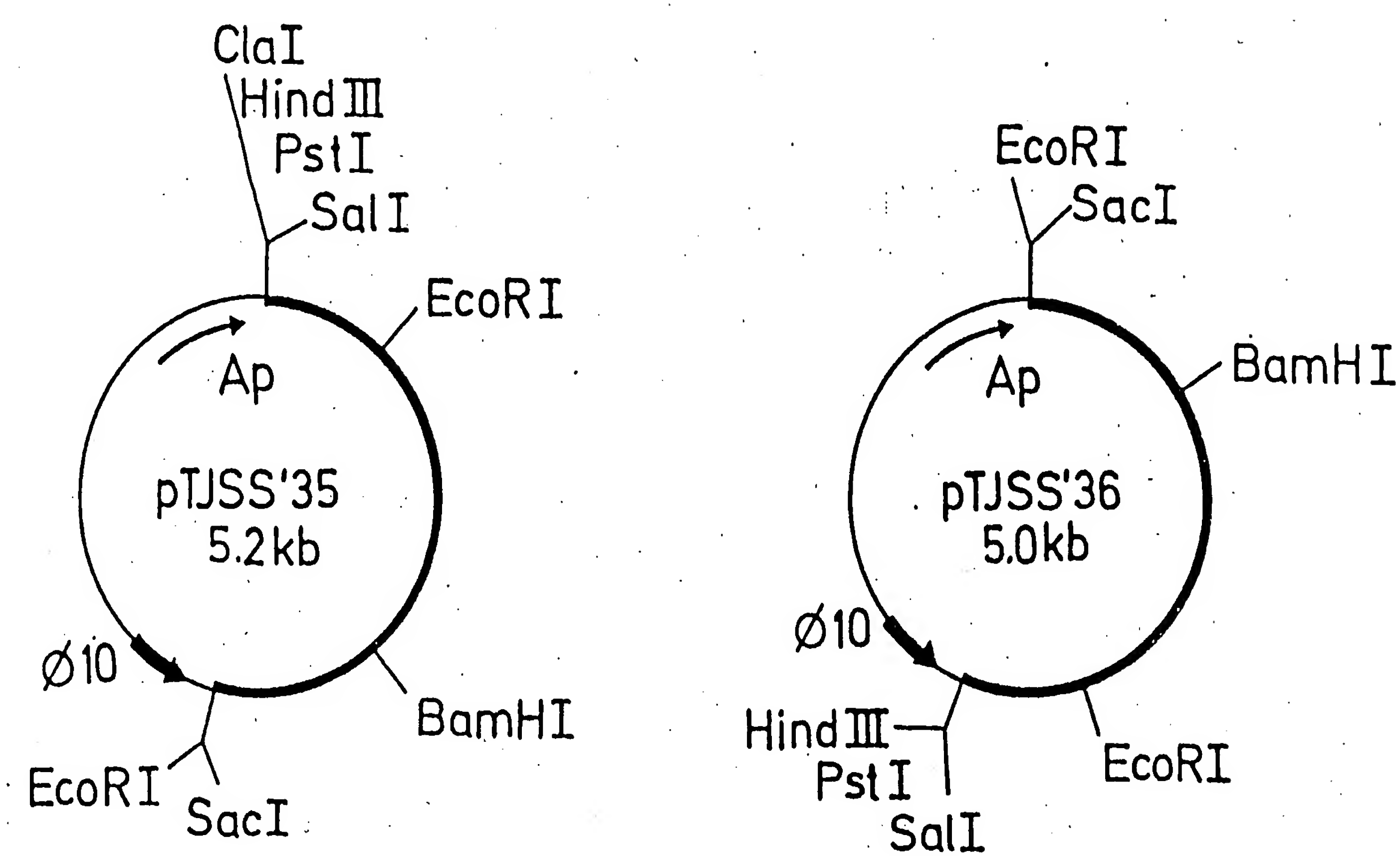


FIG. 4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SalI
EcoRI

FIG. 5

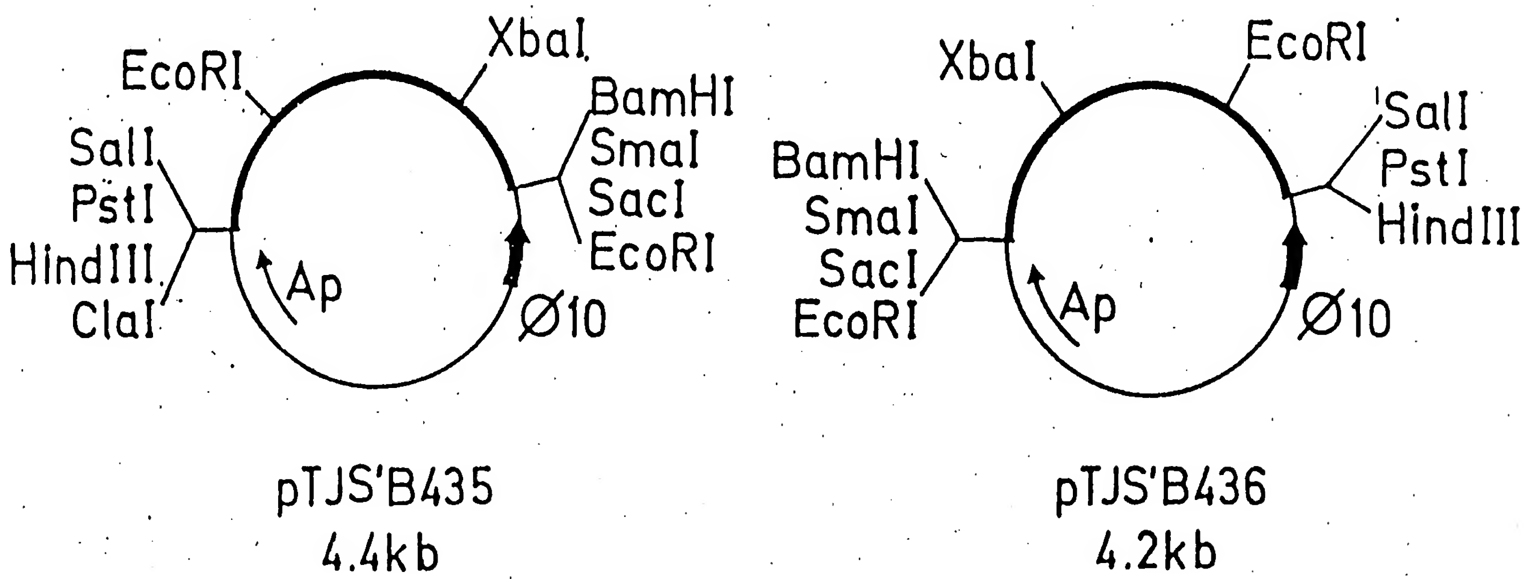
EcoRI
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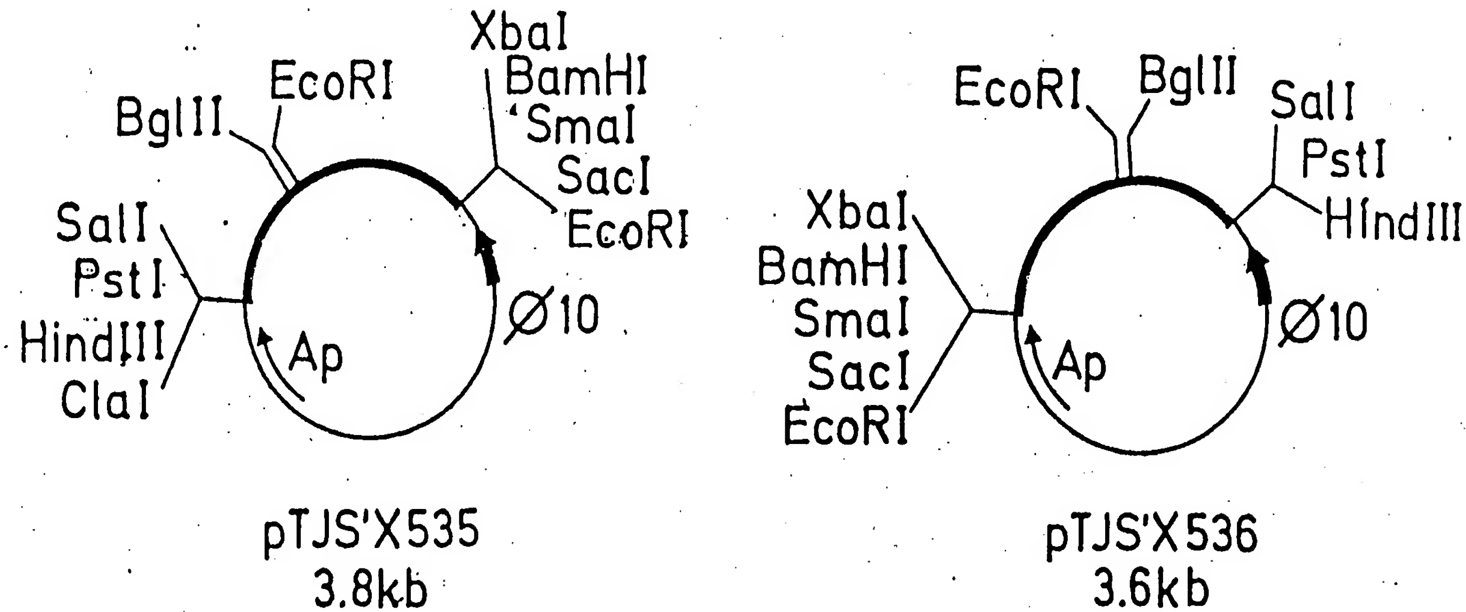
FIG. 6



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SacI
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ClaI
Ap
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3.8kb
EcoRI
BglII
SalI
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Ø10
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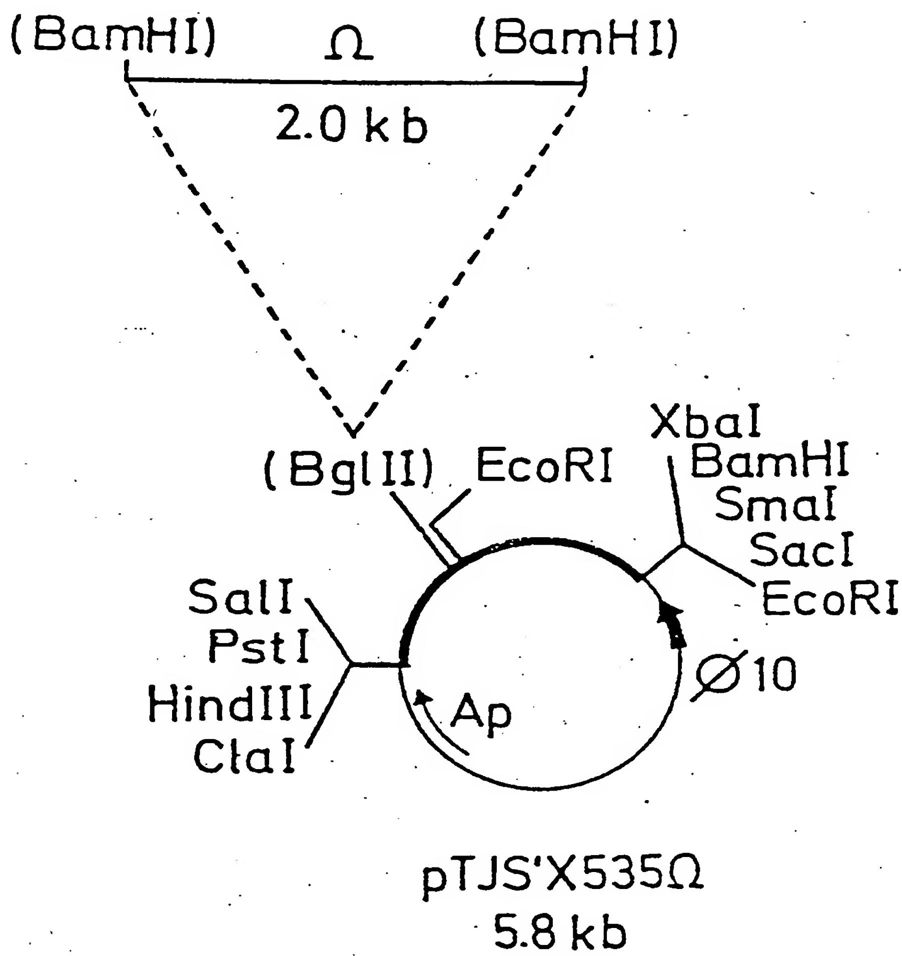
FIG. 7a



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Ω
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FIG. 7b

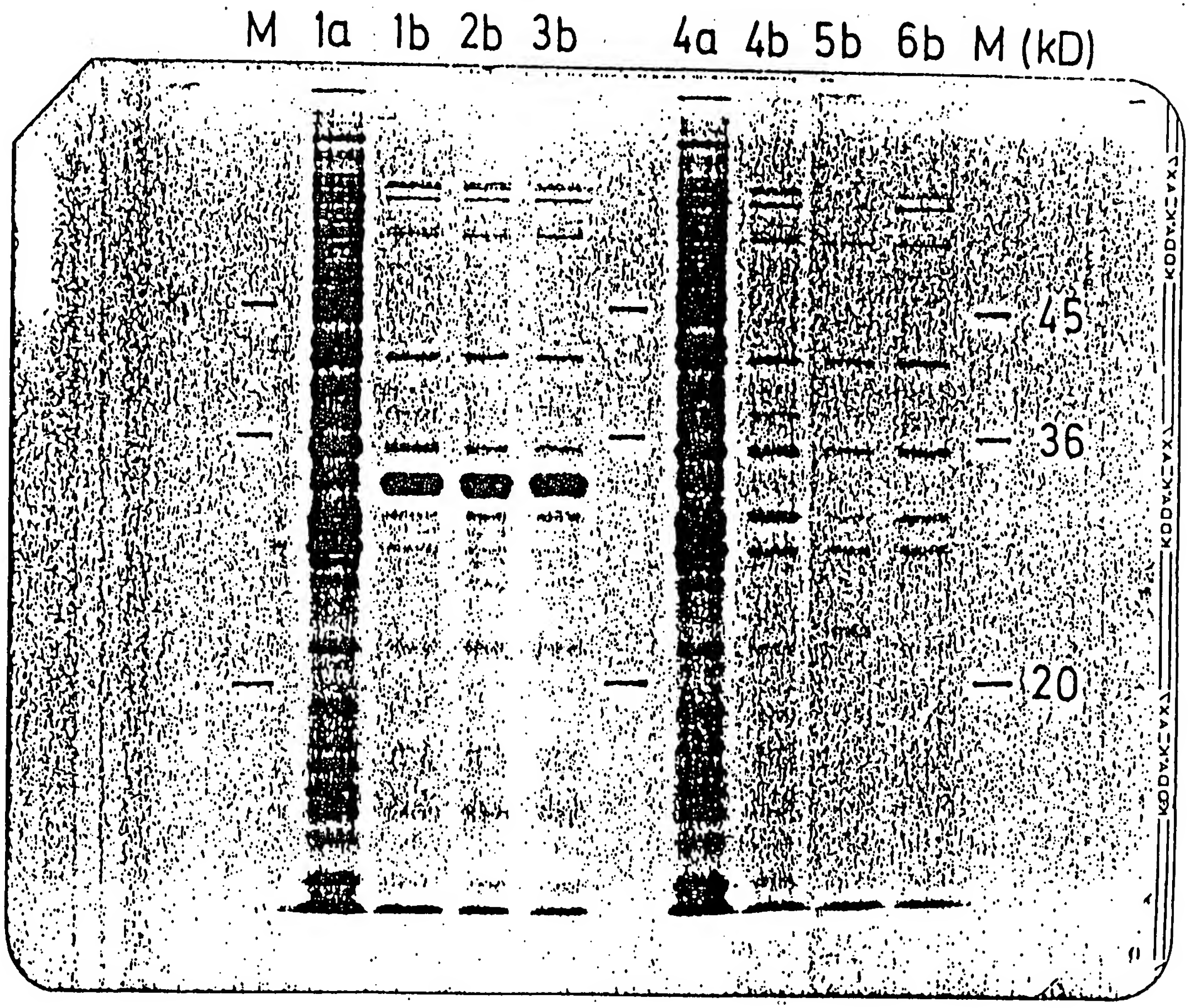

FIG. 8

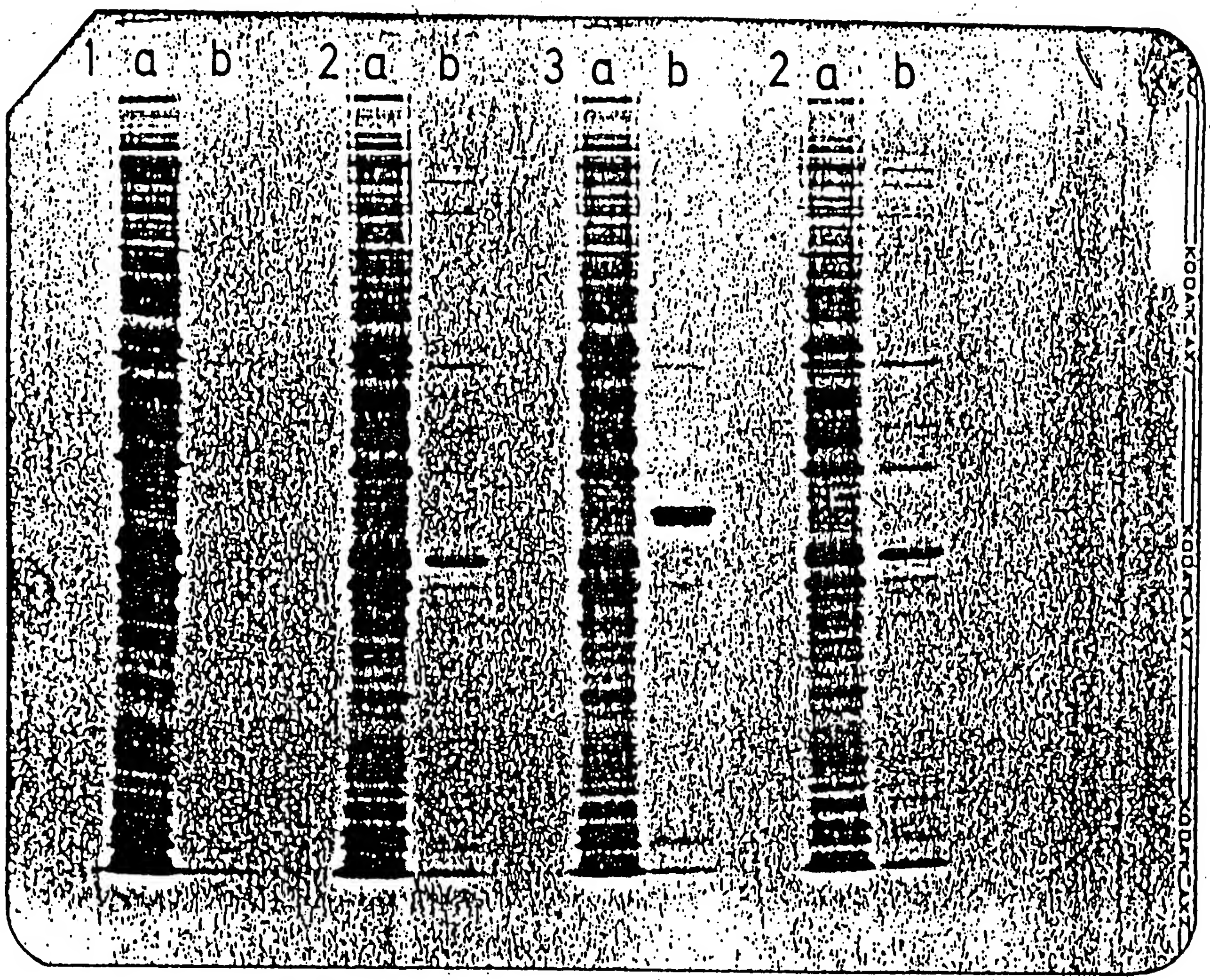

FIG. 9